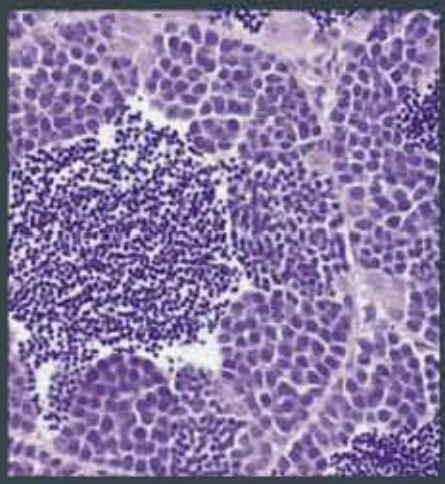
Fish Endocrinology



VOLUME 2

Editors
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Preface

During the past two decades, fish endocrinology has witnessed exciting developments due to our increased knowledge at all levels of biological organization, including molecular biology, cell biology, physiology and behaviour. New insights into development, neurobiology, immunology and molecular genetics closely correlate with classical aspects of endocrinology. The purpose of this book is to overview major advances in numerous research areas of fish endocrinology.

Larvae of bony fish are the smallest independently functioning vertebrates and achieve dramatic rates of growth during early life. Thereby they undergo gradual morphological and physiological transformations in order to develop from the larval to the juvenile and finally to the adult stage. The endocrine systems that regulate the developmental and growth processes are thus of crucial importance. Section 1 deals with the growth hormone (GH)/insulin-like growth factor (IGF) system, a research field of rapidly growing importance and impact on future research. The section starts with a survey on the IGF system and the related insulin system in mammals (1). This chapter, thought to serve as a general introduction, is followed by chapters on insulin (2, 3) and on the IGF hormones (4) IGF and insulin receptors (5) and IGF- binding proteins (6) in fish. These chapters consider the roles of the different components of the insulin-IGF family in crucial physiological processes such as metabolism, development, growth, reproduction and osmoregulation.

Particular sections of the book concentrate on classical fields of endocrine research. One is the gastro-entero-pancreatic (GEP) system, discussed in detail in Section 2. The histology and ultrastructure of the fish endocrine pancreas is dealt with for the African lungfish as a representative species (7). The functional aspects of glucagon and related hormones (8) and the development of the GEP endocrince system with its

numerous hormones in fish (9) are described. The other classical challenge for endocrinologists, the hypothalamus-pituitary axis with its great importance as the central regulator of the hormone system, is covered in Section 3. This section includes general morphofunctional and developmental aspects of the bony fish anterior pituitary (10), the proopiomelanocortin (POMC) related peptides which interact in many physiological systems (11), the osmoregulatory actions of hypophyseal hormones in general (12), and prolactin (PRL) with its central role in freshwater adaptation in particular (13).

Further topics explore the evolution, biology and function of the natriuretic hormone (NP) family, i.e., ANP, BNP and CNP (14), cardiac nitric oxide (NO) signalling (15) and the structure-activity relationships and myotropic actions of numerous peptides, such as tachykinins, bradykinins, and the neuropeptide Y (NPY) family, endothelin, vasoactive intestinal polypeptide (VIP) and galanin (16). Furthermore, the role of the fish pineal gland with its neuroendocrine messenger melatonin as a significant part of a complex time-measuring system is considered (17). These subjects are certainly challenges for future research.

Numerous bony fish species such as salmon, seabream and tilapia have high commercial value and, thus, research results on the regulatory mechanisms involved in growth, development, reproduction and stress response have a major impact not only on the science of fish biology but also on the aquaculture industry. Accordingly, these aspects are not only considered in Section 1 but also in some chapters of Section 8 dealing with the neuroendocrine control of reproduction in fish and the synthesis and functions of estrogens (18, 19), stress response (20), the hypothamus-pituitary-adrenal (HPA) axis and the roles of corticosteroids in ionic and osmotic balance, metabolism, and stress as well as in development, reproduction, and aging (21, 22). Furthermore, the influence of thyroid hormones on growth, development, parr-smolt transformation and reproduction is dealt with (23).

Development, growth and reproduction are regulated by the integration of environmental influences such as food availability, temperature and season, with endogenous neuroendocrine and endocrine signals. For several years there has been severe concern that various hormonally active chemicals designated as endocrine disrupting compounds (EDCs) which are present in surface waters and aquatic sediments, may adversely affect the development, growth, reproduction

and immune competence of fish. Several chapters (6, 19, 22, 23 and 24) are dedicated to the endocrine disruption of the growth, reproductive and immune systems.

As one may expect, creating sections in a multi-author work will cause inevitable separations of related hormones and regulatory systems, e.g., insulin is covered in the insulin – IGF chapter (2, 3) and glucagon in the portion GEP system (8). Certain aspects of neuroendocrine regulation are guven not only in section 3 but also in the chapters 4, 18, 21, 22 and 24.

The chapters in the book were peer-reviewed, besides the editors, by the following specialists: Augustine Arukwe (Ontario, Canada), Dianne Baker (Amherst, USA), Mercedes Blázquez (Barcelona, Spain), Lorenzo Colombo (Padua, Italy), Alex Eberle (Basel, Switzerland), Sture Falkmer (Trondheim, Norway), Luis Filgueira (Crawley, Australia), Olivier Kah (Rennes, France), Sakai Kikuyama (Tokyo, Japan), Werner Kloas (Berlin, Germany), Duncan MacKenzie (Texas, USA), Stephen McCormick (Turners Falls, USA), Tom Moon (Ottawa, Canada), Andreas Oksche (Gisesen, Germany), Brian C. Peterson (Stoneville, USA), Erika Plisetskaya (Seattle, USA), Gustavo Somoza (Buenos Aires, Argentina), Yoshio Takei (Tokyo, Japan), Mathilakath M. Vijayan (Waterloo, Canada) and James R. Wright (Nova Scotia, Canada). These experts contributed time and expertise and we would like to thank all of them.

The subjects covered in the book reflect the newer areas of endocrinology as well as the traditional approach to the subject. An obvious trend is that shifts the earlier focus on central control mechanisms which lead to endocrine pathways of regulation towards greater considerations of peripheral paracrine/autocrine mechanisms. Most of the chapters not only review and discuss the state-of-the-art in the respective field, but also show perspectives of future research. Consequently, most chapters end with an epilogue that draws final conclusions and tries to anticipate future trends.

We hope that the book will be of interest to a broad readership of scientists involved in basic fish research, comparative endocrinology, fisheries and aquaculture as well as for students of fish biology.

Manfred Reinecke, Giacomo Zaccone and B.G. Kapoor

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SECTION 4

Natriuretic Peptides

CHAPTER

14

The Natriuretic Peptide System of Fishes: Structure, Evolution and Function

John A. Donald* and Tes Toop

ABSTRACT

The natriuretic peptide (NP) system of fishes ranges in complexity from a single NP in cyclostomes and elasmobranchs (primarily CNP) to a complex family of seven peptides in bony fishes (ANP, BNP, VNP, and four CNPs). The identification of the peptides in bony fishes has been generated by analysis of genomic databases, and it is possible that further analysis of new databases will lead to the identification of novel NPs and receptors. The challenge for fish endocrinologists, physiologists and biochemists will be to clarify the respective roles of the different peptides and their receptors in different species and to make sense of the additional complexity. Meanwhile, the current data uphold the original premises, that the NP system of fishes is involved in both cardiovascular and osmotic regulation. This chapter reviews our current knowledge of the structure and function of the NP system in fishes.

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Key Words: Natriuretic peptides; Natriuretic peptide receptors; Fishes; cGMP; Osmoregulation; Cardiovascular physiology; Brain.

Abbreviations: ANP-atrial natriuretic peptide; BNP-brain natriuretic peptide; CNP-C-type natriuretic peptide; cAMP-adenosine 3', 5' cyclic monophosphate; cGMP guanosine-3', 5' cyclic monophosphate, CFTR cystic fibrosis transmembrane regulator, EbuNP *Eptatretus burgeri* NP; GC-guanylyl cyclase; mRNA-messenger ribonucleic acid; NPR-natriuretic peptide receptor; NPs-natriuretic peptides; sCP-salmon cardiac peptide; VIP-vasoactive; intestinal peptide; VNP-ventricular natriuretic peptide.

INTRODUCTION

The natriuretic peptide (NP) system of fishes has been studied for over 15 years and the knowledge base on this system in vertebrates is only greater for mammals. In fact, the earliest fish studies on the NPs dates to the mid-1980s (e.g., O'Grady et al., 1985; Solomon et al., 1985; Duff and Olson, 1986), which is only a few years after the original discovery of atrial natriuretic factor (= atrial natriuretic peptide, ANP) in rat cardiac myocytes by de Bold and colleagues (1981). What is interesting about these early studies in fishes, which are all physiological, is that they set up the original paradigms for NP functions in fishes that have remained essentially unchallenged since. In mammals, the fact that the system is protective of the cardiovascular system, reducing blood pressure by a variety of primary and secondary effects on the vasculature and kidneys (see Samson and Levin, 1997), has led to the hypothesis that the system could function in regulating fish osmotic and cardiovascular homeostasis. The system now appears to have a variety of functions in mammals, including roles in development, immunity and reproduction (see Samson and Levin, 1997), and such potential functions are starting to be examined in fishes. More recently, with the advent of fish genome projects, mapping the evolution of the system in non-mammalian vertebrates has produced some fascinating hypotheses on the phylogeny of the system (e.g., Inoue et al., 2003a). This chapter, therefore, concentrates on what is understood about the structure and function of the system in fish in relation to cardiovascular and osmoregulatory function and offers some insight into the relationship of the system in the different classes of fishes.

Different fishes use different physiological strategies in order to maintain osmotic homeostasis in the face of hyperosmotic and

hypoosmotic challenges. The hagfishes are the only group that display little osmotic strategy because they are entirely marine, and behave similarly to marine invertebrate osmoconformers. They maintain their extracellular fluid at a concentration that is slightly hyperosmotic to the surrounding seawater, which is typical of marine osmoconformers (Riegel, 1998). It is unclear, therefore, whether the clearly well-developed NP system of hagfishes functions solely in cardiovascular regulation, or whether there are additional functions. Chondrichthyan fishes are mainly a marine group, although there are euryhaline and even freshwater representatives (Karnaky, 1998). Marine chondrichthyans maintain extracellular NaCl concentrations at a level lower than seawater but, like hagfishes, maintain an internal environment slightly hyperosmotic to the external medium. However, the extracellular osmolality is not made up entirely of inorganic ions but is maintained principally because of high urea concentrations. These fishes, therefore, must regulate NaCl by countering passive ionic influx with active transport of salt out of the organism via the rectal gland, while the kidney must reabsorb filtered urea (Karnaky, 1998; Hyodo et al., 2004). Study of the NP system of chondrichthyans has largely focused on the effects of NPs on rectal gland function, although there are also some data on branchial, renal and cardiovascular aspects.

Most osteicthyan fishes are totally marine but many live exclusively in freshwater, while fewer are completely euryhaline and are able to survive in either freshwater or salt water (for example, anguillids and salmonids). In the freshwater environment, bony fishes must maintain their internal ionic concentrations above that of the environment. They face a net water influx and ion loss that must be counteracted by active uptake of salt from the environment, primarily across the gills, and loss of water across the kidney. The renal tubule also acts to reabsorb salt from the primary urine and a copious hypoosmotic urine is excreted (Karnaky, 1998). In the marine environment, the vectors for salt and water are the opposite to that of freshwater. Fish must counter dehydration as seawater is more concentrated than the internal body fluids, while salt is gained passively because of higher external salt concentrations. The intestine, gills and kidneys are the principle osmoregulatory tissues involved in seawater (SW) osmoregulation. Following SW drinking, salt, and secondarily water, are taken up across the gut. The salt uptake is counteracted by active branchial excretion. The kidney is unable to concentrate urine and is therefore unable to offload salt in this manner, but filtered water is

reabsorbed secondarily to salt, and thus scant isotonic urine is produced. Alternatively, some marine teleosts lack glomeruli and all urine produced is via a secretory mechanism. The urinary bladder supplies an additional location for the reabsorption of water and this is subsequent to the reabsorption of NaCl (Karnaky, 1998). Studies of the osmoregulatory effects of NPs in bony fishes have targeted all these osmoregulatory organs; however, much of the data are not definitive on the precise mechanisms by which NPs facilitate osmoregulation in the relevant tissues. It is worth noting that the lamprey (Class Agnatha) is euryhaline and, as far as is known, osmoregulates in a manner analogous to bony fishes in both environments (Karnaky, 1998). There is very little information on the role of NPs in the lamprey.

CARDIAC NATRIURETIC PEPTIDES

Our understanding of the types and molecular evolution of NPs has increased dramatically with the advent of fish genomic databases such as the puffer fish, *Takifugu rubripes*, and the medaka, *Oryzias latipes*. As an example, the recent paper of Inoue et al. (2003a) was able to use chromosome analysis in fishes and humans in order to construct a phylogenetic lineage describing the evolution of NPs. Undoubtedly, such approaches will continue to provide new insights on the types of NPs in fishes and other non-mammalian vertebrates. All mature NPs are C-terminal peptides derived from separate genes. They show structural homology due to a disulphide bridge formed by two cysteine residues flanking a sequence of 17 amino acids that shows homology across the vertebrates (Fig. 14.1).

Agnathans

Recently, the first cloning and sequencing of a cardiac NP from a cyclostome has been performed in the hagfish, *Eptatretus burgeri* (Kawakoshi et al., 2003). The peptide was found to be a novel NP and was called EbuNP (Fig. 14.1). Importantly, no other type of NP was found in the heart after specific removal of EbuNP mRNA from the mRNA pool used for cDNA synthesis. Analysis of the EbuNP sequence showed that it shared the characteristics of each known type of NP. The intramolecular ring sequence showed the highest identity to CNP, but the peptide contained a ten-amino acid tail following the second cysteine, which is not found in CNP (Fig. 14.2). EbuNP mRNA was strongly expressed in both

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EbuNP          GSTSDG CFGVKMDRIGASTGLGC RGARRRTFS
TriakisCNP     G-PSRG CFGVKLDRIGAMSGLGC
FuguANP        -KRASS CFGARMDRIGNASGLGC NNGR
FuguBNP        -RRSSS CFGRRMDRIGSMSSLGC NTVGKYNPK
FuguCNP1       G-WNRG CFGLKLDRIGSMSGLGC
EelVNP         -KSFNS CFGTRMDRIGSWSGLGC NSLKNGTKKKIFGN
ToadANP        ---SSD CFGSRIDRIGAQSGMGC G--RRF
RanaBNP           SN CFGRRIDRIGDVSGMGC NGSRNRYP
RanaCNP1       G-YSRG CFGVKLDRIGAFSGLGC
ChBNP          MMRDSG CFGRRIDRIGSLSGMGC NGSRKN
ChCNP          G-LSRS CFGVKLDRIGSMSGLGC
HumanANP       SLRRSS CFGGRMDRIGAQSGLGC NSFRY
HumanBNP    SPKMVQGSG CFGRKMDRISSSSGLGC KVLRRH
HumanCNP       GLSKG CFGLKLDRIGSMSGLGC
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Fig. 14.1 Alignment of selected mature NPs from each vertebrate class showing the sequence homology (asterisks) within the seventeen amino acid ring formed by a disulphide bridge between cysteine residues (boxed), which is diagnostic of NPs. Across the classes, nine amino acids are conserved within the ring. The NPs and their accession numbers are as follows: *Eptatretus burgeri* NP- EbuNP (AB087732); *Triakis scyllia* (Triakis) CNP (AB047081); *Takifugu rubripes* (Fugu) ANP (AB089933); *Takifugu rubripes* (Fugu) BNP (AB089934); *Takifugu rubripes* (Fugu) CNP-1 (AB089933) (AB089935); *Anguilla japonica* (Eel) VNP (AB019371); *Bufo marinus* (Toad) ANP (AF429999); *Rana catesbeiana* (Rana) BNP (Fukuzawa et al., 1996); *Rana catesbeiana* (Rana) CNP-1 (D17413); *Gallus domesticus* (chicken) BNP (X57702); *Gallus domesticus* (chicken) CNP (P21805); human ANP (NP_006163); human BNP (NP_002512); human CNP (NP_077720).

the systemic and portal hearts, and weakly expressed in the intestine, but no expression was detected in the gill and kidney. The processed form of EbuNP in the heart and plasma was found to be a 68 amino acid peptide with an amidated C-terminus like *Anguilla japonica* ANP, and it was concluded that the peptide is most likely cleaved by the processing enzyme, furin, as is BNP but not ANP or CNP (Kawakoshi et al., 2003). The only NP cloned from the heart of lampreys is a CNP, which was obtained from *Lampetra japonica* and *Geotria australis* (Kawakoshi, A., Hyodo, S., Stower, S. and Takei, Y., unpublished).

Chondrichthyes

There is now very strong evidence that chondrichthyan fishes only have one NP in the heart and brain, namely CNP (Fig. 14.3). Suzuki et al.

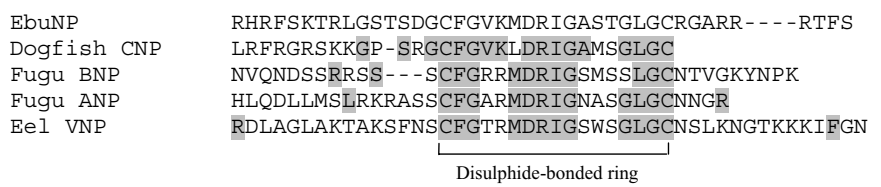

Fig. 14.2 Alignment of 41 amino acids from the C-terminal of the deduced amino acid sequence of the NP (EbuNP) cloned from the heart and brain of the hagfish, *Eptatretus burgeri*. The shading indicates conserved residues between EbuNP and dogfish CNP, Fugu BNP, Fugu ANP and eel VNP. EbuNP shows most similarity to dogfish CNP, but has a C-terminal tail, and conserved amino acids with Fugu ANP and BNP and eel VNP.

(1991, 1992) isolated a 115 amino acid proCNP from the heart of two species, *Scyliorhinus canicula* and *Triakis scyllia*, but could only isolate CNP-22 from the brain (see below), which suggested that CNP is processed differently in the heart and brain. Concurrently, a CNP cDNA was cloned from the heart of the spiny dogfish, *Squalus acanthias* (Schofield et al., 1991). Both studies showed that the CNP-22 of elasmobranchs was very similar to other vertebrate CNPs. Recently, a CNP cDNA was cloned from the heart of *T. scyllia*, and when the CNP mRNA was knocked out of the mRNA pool, only truncated CNP cDNAs were amplified. Thus, it was concluded that the heart only expresses CNP from a single gene (Kawakoshi et al., 2001). Comparison of the three pro-CNP sequences shows a remarkable similarity between species (Fig. 14.3). In fact, there are only three amino acids differences between proCNP-115 of *T. scyllia* and *S. canicula*, and the mature CNP-22 is identical between the two species. In contrast, proCNP-115 of *S. acanthias* shows more variation and there are two amino acid differences in the mature CNP-22 of *S. acanthias* compared to those of *T. scyllia* and *S. canicula*. This is unusual given the conservation in proCNP in elasmobranchs and the fact that CNP is the most conserved NP in vertebrates. In *T. scyllia*, a homologous radioimmunoassay found that detectable levels of CNP could be found in peripheral tissues such as the kidney, liver, and digestive tract; in addition, CNP was found in the plasma at a concentration of 1.97 $pmol.ml^{-1}$ (Suzuki et al., 1994). In Rajiformes, CNP cDNAs have been cloned from the heart of three species, and the mature CNP-22 of each species is identical to that of *T. scyllia* and *S. canicula* (Hyodo, S., Kawakoshi, A., Bartolo, R., Takei, Y., Toop, T. and Donald, J; unpublished).

Fig. 14.3 Alignment of proCNP of three elasmobranch species illustrating the remarkable homology between the prohormones. *Triakis scyllia* and *Scyliorhinus canicula* only have 3 amino acid differences (shaded residues) and show 97.4% similarity. The asterisks indicate homology between the three species and show that proCNP of *Squalus acanthias* is more variant compared to *Triakis* and *Scyliorhinus*.

Osteichthyes

The NP family of the bony fishes is the most complex of any vertebrate class studied to date (Fig. 14.4). This finding is not especially surprising since the genomes of bony fishes, particularly teleost genomes, are peculiarly plastic, demonstrating an expansion in the number of genes in many gene families (Robinson-Rechavi et al., 2001). The bony fish NP family has four members (Kawakoshi et al., 2004). The earliest NP to be isolated was ANP from the eel, *A. japonica* (Takei et al., 1989), and since then ANP has been cloned from the heart of *A. japonica* (Takei et al., 1997a), killifish, *Fundulus heteroclitus* (Takei and Hirose, 2002), tilapia, *Oreochromis mossambicus*, pufferfish, *T. rubripes*, and sturgeon, *Acipenser transmontanus* (Kawakoshi et al., 2004) (Fig. 14.4). In the following discussion, the common name eel will refer to *A. japonica*; other eel species will be referred to by their scientific names. The carboxyl terminal of the ANPs end with a glycine residue, which is a signal for C-terminal amidation. Carboxyl terminal amidation of circulating eel ANP and EbuNP has been demonstrated by HPLC-mass spectrometry (Takei et al., 1997a; Kawakoshi et al., 2003), and it is highly likely that other circulating fish ANPs terminating with a glycine are likewise amidated; such fish ANPs include those of *T. rubripes*, *O. mossambicus*, *F. heteroclitus* and *A. transmontanus*. A different type of cardiac peptide is found in salmonids, which has been named trout ANP or salmon cardiac peptide (sCP), depending on the species in which it was isolated (Takei et al., 1997b; Tervonen et al., 1998). The two sequences differ by only one amino acid and, thus, should be considered homologues. Although the salmonid cardiac peptides are not amidated, they demonstrate greatest similarity with the cDNAs and prohormones of *O. mossambicus* and *F. heteroclitus*

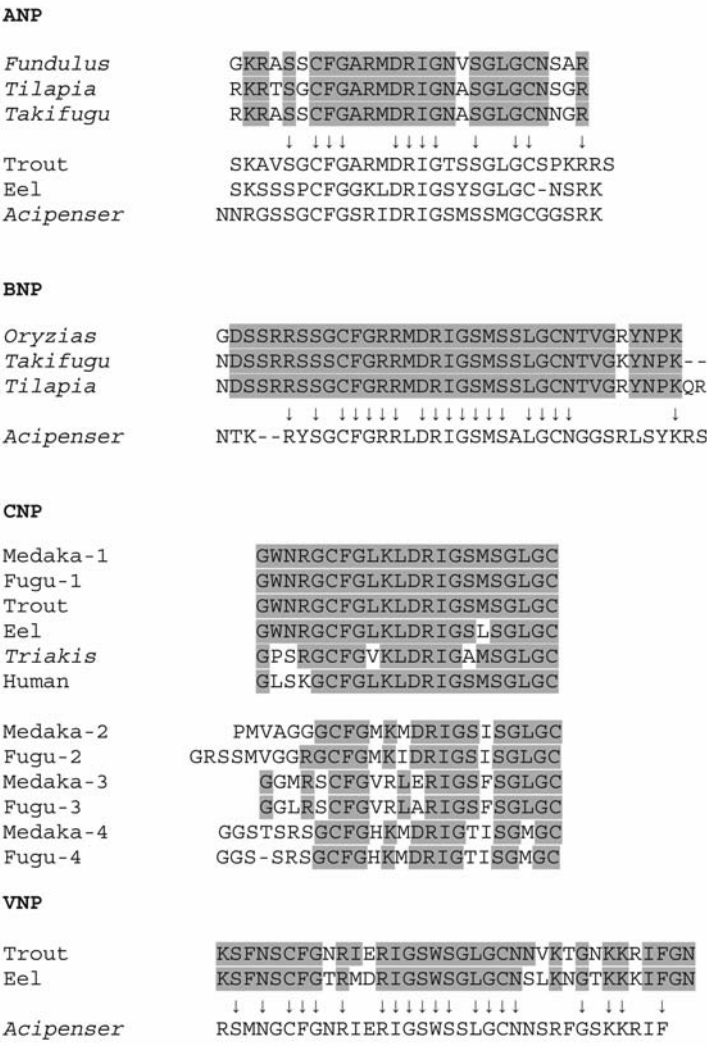

Fig. 14.4 Alignment of the known NPs from bony fishes. For ANP, the shading shows conserved amino acids between *Fundulus heteroclitus*, *Tilapia mossambicus*, and *Takifugu rubripes*, and the arrows show the degree of amino acid conservation across each species. The glycine residue that is an amidation signal has been omitted from the C-terminal of *Fundulus*, *Tilapia*, *Takifugu*, eel and *Acipenser* ANP; it is known that eel ANP is amidated during post-translational processing Takei et al., 1997a). For BNP, the shading demonstrates conserved amino acids between *Tilapia mossambicus* and *Takifugu rubripes*, and the arrows show the degree of amino acid conservation between those species and *Acipenser transmontanus*. For CNP, the shading shows the degree of homology between CNP-1 of *Oryzias latipes* (medaka) and *Takifugu rubripes* (fugu) and CNP of trout, eel, *Triakis scyllia*, human, and CNP-2, -3, and -4 of *Oryzias* and *Takifugu*. For VNP, the shading shows conserved amino acids between trout and eel, and the arrows show the degree of amino acid conservation between those species and *Acipenser transmontanus*.

ANP (Takei and Hirose, 2002), which indicates that they are the salmonid form of ANP.

In common with all NPs, mature osteicthyan ANP (27-29 amino acids) contains an intramolecular seventeen member amino acid ring formed by a disulfide bridge between two cysteines. Mature ANP has six N-terminal amino acids (seven in *A. transmontanus*) before the ring and between four and six C-terminal amino acids following the ring (Kawakoshi et al., 2004). Like VNP (see below) and CNP, ANP is synthesized as a preprohormone from which the signal sequence is removed to form the prohormone; the mature peptide is cleaved from the C-terminal of the prohormone (Loretz and Pollina, 2000). The majority of circulating eel ANP and sCP is in the form of the mature peptide (Takei et al., 1997a; Kokkonen et al., 2000). The most potent secretagogue for ANP in eels is plasma osmolality, with volume expansion being less potent (Kaiya and Takei, 1996). In rainbow trout, *Onchorhyncus mykiss*, distension of the heart induced ANP release (Cousins and Farrell, 1996). Using a perfused ventricle preparation, Kokkonen et al. (2000) demonstrated that both mechanical load and endothelin-1 stimulate sCP release from the perfused ventricles of Atlantic salmon, *Salmo salar*, as measured by sCP immunoreactivity of the perfusate. The tissue distribution of fish ANPs has been studied in the eel, and RT-PCR has demonstrated expression in the brain, heart, gill, intestine, red body, head kidney and kidney (Takei et al., 1997a). In contrast, salmon cardiac peptide is only expressed in the atrium and ventricle (Tervonen et al., 1998; Majalahti-Palviainen et al., 2000).

Bony fishes also possess a novel peptide, ventricular NP (VNP), which has been sequenced from the ventricle of trout, eel (Takei et al., 1991, 1994a,b; Takei, 2000a) and *A. transmontanus* (Kawakoshi et al., 2004) (Fig. 14.4). This peptide is extended at the carboxyl terminus compared with ANP and BNP but has only five amino acids preceding the intramolecular ring (Fig. 14.3). Until recently, it was thought that VNP was the piscine form of BNP, but it has since been discovered that BNP as well as VNP is present in the heart of osteicthyans. To date, BNP cDNAs have been cloned and sequenced from *T. rubripes*, *A. transmontanus*, *O. mossambicus* (Kawakoshi et al., 2004), and *O. latipes* (Inoue and Takei, accession no. AB099700.1). BNPs are characterized by having a longer amino terminal tail than the other members of the NP family and a carboxyl terminal tail that is intermediate in length between those of ANP

and VNP (Fig. 14.3). In addition, BNPs have the characteristic AUUUA motif repeated in the 3' untranslated region of the mRNA transcript, as well as two consecutive arginines within the intramolecular ring (Kawakoshi et al., 2004). The secretagogues for piscine BNP and the circulating form are currently unknown. However, eel VNP circulates mainly as its high molecular weight form (VNP 1-36), although a C-terminally truncated form (VNP 1-25) also circulates. In addition, the prohormone makes up approximately one third of plasma VNP immunoreactivity (Takei et al., 1994c). In eels, VNP responds to the same stimuli for secretion as ANP, namely increased plasma osmolality and volume load (Kaiya and Takei, 1996). Northern blotting located the expression of eel VNP mRNA in the atrium and ventricle. Unlike ANP, the major site of expression is the ventricle (Takei et al., 1994b). The intestine is also a site of synthesis of both ANP and VNP in the eel. The presence of these peptides was demonstrated using homologous and non-cross-reacting antibodies to ANP and VNP and also by RT-PCR (Loretz et al., 1997). Immunohistochemistry showed that ANP and VNP immunoreactivity was confined to the epithelial cells and was present in all segments of the intestine. In the chondrostean, *A. transmontanus*, the relative tissue distribution of ANP, VNP and BNP mRNA transcripts, as measured by RT-PCR, is interesting. ANP mRNA was abundantly expressed in the atrium and considerably in the ventricle but not in the brain. BNP was expressed abundantly in both the atrium and ventricle with a considerable amount also being present in the brain. VNP was expressed to the same extent in both chambers of the heart and very slightly in the brain. Interestingly, no mRNA transcripts for the peptides were found in the gill, kidney or intestine (Kawakoshi et al., 2004).

Analysis of the genomes of *T. rubripes* and *O. latipes* has led to the identification of four CNPs in these species, which are designated CNP-1, CNP-2, CNP-3, and CNP-4 (Inoue et al., 2003a; Fig. 14.4). CNP-1 is most similar to CNP of elasmobranchs, eel and tetrapods. In *O. latipes*, CNP-1 and CNP-2 are exclusively expressed in the central nervous system, whereas CNP-3 and CNP-4 are expressed in a range of tissues in the periphery including the heart (Inoue et al., 2003a). Recently, CNP was cloned from eel, and a homologous radioimmunoassay showed high levels of CNP in the heart and plasma of freshwater (FW) eels, which suggests that CNP is an important circulating hormone in eel (Takei et al., 2001). In the same study, tissue mRNA expression was measured by both RNAse protection assay and RT-PCR. With the former, the brain contained the

most transcript followed by the atrium, ventricle and liver. However, using RT-PCR, the gills, digestive tract and kidneys also showed some expression. In trout, RT-PCR analysis demonstrated mRNA expression in the brain with a small amount of transcript being found in the atrium but not in other tissues (Inoue et al., 2003b).

Molecular Evolution of Fish Natriuretic Peptides

The analysis of the known fish NP sequences has led to the hypothesis that CNP is the ancestral peptide. CNP is the most conserved of the NPs and is the only NP found in elasmobranchs (Kawakoshi et al., 2001). Based on this evidence and the chromosomal analysis presented by Inoue and co-workers (2003a), CNP is believed to be closest to the ancestral form of the peptide; although EbuNP is equally similar to ANP, BNP, VNP and CNP, but unlike CNP it has a C-terminal extension (Fig. 14.2; Kawakoshi et al., 2003). Kawakoshi and colleagues hypothesize that either the arginine residue immediately following the intramolecular ring of EbuNP underwent a single-point mutation to generate a stop codon to form the ancestral CNP of the other vertebrate groups, or that a mutation occurred in the ancestral vertebrate CNP to continue the sequence at the carboxyl end of the peptide. The analysis of the four CNPs cloned from *O. latipes* and *T. rubripes* indicate that CNP-1 is the CNP most similar to the CNP sequence published for trout and eel (Inoue et al., 2003a). CNP-3, not CNP-1, is the CNP associated with chondrichthyans and frog CNP-1, according to the nomenclature of Inoue et al. (2003a). CNP-1 and CNP-2 are lost in all but the osteicthyan lineage and CNP-4 is the CNPII of frogs and the CNP in mammals. CNP-3 underwent tandem duplications before the osteicthyan divergence to form the ANP and BNP of fishes and tetrapods, the evidence for which is demonstrated in another species of puffer fish, *Tetraodon nigrovirdis*, where CNP-3 is tandemly located with ANP and BNP on the same chromosome (Inoue et al., 2003a). Presumably, this is true in amphibians that retain CNP-3 as frog CNP-I, and they also have ANP and BNP. The evolutionary history of piscine VNP, which has yet to be found in tetrapods, remains unknown.

NATRIURETIC PEPTIDE RECEPTORS

Like other peptide hormones, NPs must bind to and activate membrane-bound receptors (NPR) to elicit cellular and physiological responses (Table 14.1). In fishes, as in mammals, NPs interact with two classes of

Table 14.1

Class of fish	*Kidney*	*Gills*	*Rectal Gland*	*Head kidney/adrenals*	*Gut*	*Heart and Blood Vessels*
Agnatha	ANP-and CNP-binding sites on glomeruli and archinephric duct (hagfish)	NPR-GC mRNA expression ANP and CNP binding sites Vasodilation	NA	ND	ND	ANP-binding sites on ventral aorta (hagfish) Vasodilation
Chondrichthyes	NPR-B mRNA expression ANP-mediated antidiuresis in shark	CNP-binding sites NPR-C mRNA expression NPR-B mRNA expression: ND	NPR-B mRNA expression CNP ↑Cl secretion in rectal gland	ND	ND	Vasodilation Biphasic pressor and depressor response
Osteichthyes	NPR-A, NPR-B, NPR-C mRNA expression ANP binding sites ANP-mediated diuresis, natriuresis; antidiuresis in SW eel	NPR-A, NPR-B NPR-C, NPR-D mRNA expression ANP binding sites Vasodilation ANP: ↑ Na/K-ATPase	NA	ANP-binding sites: steroidogenic and chromaffin cells NPR-C ANP: ↑Cortisol secretion; ↑ Adrenaline secretion	NPR-A, NPR -B (inc. liver), NPR-C ↓Drinking, ↓NaCl absorption, ↓motility	NPR-A, NPR -B, NPR-C in heart ANP: no effect Biphasic pressor and depressor response

receptors. These are the particulate guanylyl cyclase (GC) receptors, NPR-A and NPR-B, which signal to the interior of the cell by forming the second messenger cyclic GMP (cGMP), and the non-GC receptors NPR-C and NPR-D (the latter, a novel receptor thus far identified only in the eel) (Kashiwagi et al., 1995; Loretz and Pollina, 2000; Fig. 14.5). Originally, NPR-C was thought to act as a 'clearance' receptor, modulating circulating concentrations of NPs and regulating the amount of NP available to bind to the GC receptors (Maack et al., 1987). However, it is now apparent that in mammals, NPR-C is linked to other intracellular messengers (Murthy and Makhlouf, 1999). Both the inhibition of the cyclic AMP system and the activation of phosphoinositide hydrolysis have been demonstrated, the mechanism of which appears to be via a G-protein-activating binding domain on the NPR-C (Murthy and Makhlouf, 1999). Recently, forskolin-stimulated cAMP production was found to be inhibited by ANP, CNP and C-ANF (a synthetic NPR-C specific ligand) in eels, suggesting a mechanism via the NPR-C (Fig. 14.6). Subsequent sequence analysis revealed that the eel NPR-C (and NPR-D) displays a similar G-protein-activating binding domain to that found in the mammalian receptor (Fig. 14.6; Callahan et al., 2004).

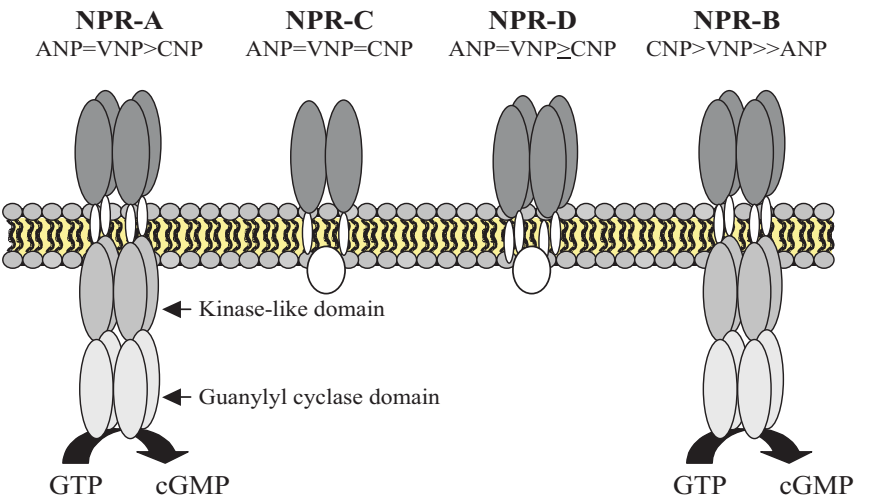

Fig. 14.5 Schematic of the structure and binding affinities of the four natriuretic peptide receptors cloned from the Japanese eel, *Anguilla japonica*. NPR-A, NPR-B and NPR-D are tetrameric receptors, whereas NPR-C is a homodimer. Both NPR-C and NPR-D have short intracellular domains. Modified from Takei and Hirose (2002). The binding affinity of piscine BNP is unknown.

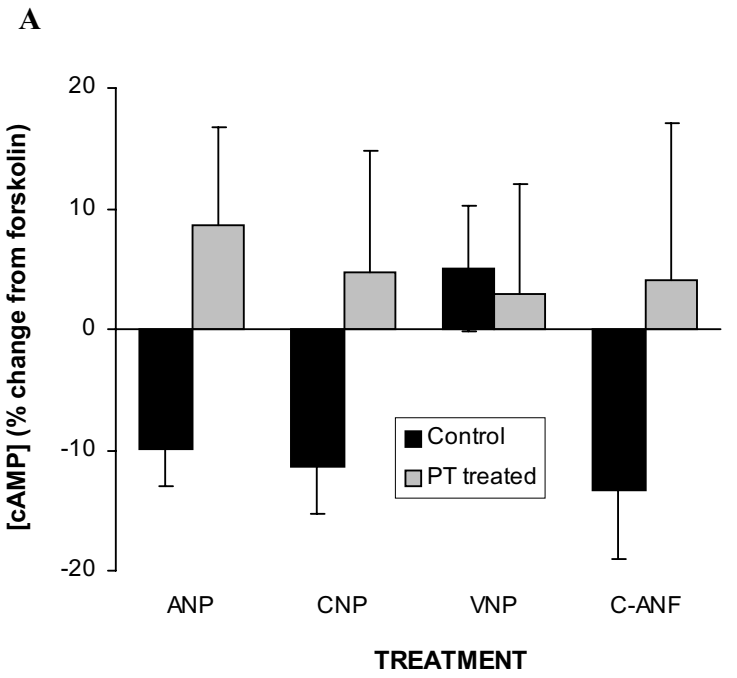

Fig. 14.6 Evidence for NP mediated inhibition of adenylyl cyclase by NPR-C in eel gill cells. **A**. Natriuretic peptide effects on forskolin-evoked adenylyl activity in dispersed eel gills cells. Amidated eel ANP, eel CNP and the NPR-C specific ligand, C-ANF, but not eel VNP significantly inhibited the forskolin-mediated stimulation of adenylyl cyclase activity. This inhibitory effect of NPs was reversed in cells pretreated with the G_i protein inhibitor, pertussis toxin (PT). **B**. Alignment of the intracellular domain of eel (*A. australis* and *A. japonica* are identical) and rat NPR-C (homology indicated by shading) showing the amino acids (bold) that represent the motif for binding and activating of G_i proteins. In addition, the homologous intracellular domain of eel NPR-D is shown since it contains a similar motif for G protein binding. Modified from Callahan et al. (2004).

Eel NPR-A and NPR-B, NPR-C and the novel NPR-D, which shares 70% sequence identity to the NPR-C, have all been cloned (Katafuchi et al., 1994; Kashiwagi et al., 1995, 1999; Takashima et al., 1995). Recently, in *O. latipes,* two homologues of NPR-A, OlGC2 and OlGC7 (Yamagami et al., 2001) and NPR-B, termed OlGC1 (Takeda and Suzuki, 1999) have been cloned, both from genomic DNA and cDNA. The mRNA of the two homologues of the NPR-A (OlGC2 and OlGC7) had similar, but not identical tissue expression. Before the advent of fish genomic studies and the recent revelations of several different subtypes of NPs and receptors (Yamagami et al., 2001; Inoue et al., 2003a; Kawakoshi et al., 2004), our knowledge of the binding affinities of the different NP ligands for the receptors greatly followed the mammalian pattern with VNP substituting for BNP (prior to the discovery of BNP in bony fishes). In order of sensitivity, eel NPR-A bound eel ANP, amidated ANP and VNP > CNP, while eel NPR-B bound eel CNP > VNP > ANP (Katafuchi et al., 1994; Kashiwagi et al., 1999; Fig. 14.5). Eel NPR-C bound eel ANP, amidated ANP, VNP and CNP with almost identical affinities (Takashima et al., 1995; Fig. 14.5), while NPR-D bound ANP, amidated ANP and VNP with high affinity (0.1-0.5 nM range), and CNP with a slightly lower affinity (Kashiwagi et al., 1995; Fig. 14.5). In sharks, NPR-B cDNA has been cloned from the rectal gland of *S. acanthias* (Aller et al., 1999), and a partial NPR-C sequence has been obtained from the gills of the same species (Donald et al., 1997). In Agnatha, an NP GC receptor has been partially sequenced from the gills of the New Zealand hagfish, *Eptatretus cirrhatus*, but it is not clear whether this is an NPR-A, NPR-B or a novel GC receptor (Callahan et al., 2000).

The tissue-specific expression of the NPRs in fishes indicates the target organs for the different NPs (Table 14.1). Eel NPR-A is widely expressed in many tissues, as measured by RNAase protection assay, including brain, gill, heart, gastrointestinal tract, liver, red body, renal system and head kidney. Interestingly, only minor differences were observed between FW and SW animals (Kashiwagi et al., 1999). The two *O. latipes* NPR-A homologues are somewhat differentially expressed in tissues. While the cDNA for both receptors is expressed in a range of tissues, the highest expression for OlGC2 was in the kidney, gall bladder and gill, and OlGC7 was expressed in these tissues and also the brain (Yamagami et al., 2001). RNAse protection assay indicated widespread expression of eel NPR-B, with the greatest expression in the liver, cardiac atrium and gill. In SW eels, these latter tissues also demonstrated a

marked decrease in the expression of NPR-B (Katafuchi et al., 1994). The expression of *O. latipes* NPR-B mRNA (OlGC1) in the brain, eye, liver and intestine was demonstrated by Northern blot, whereas RT-PCR indicated additional expression in the kidney, spleen, pancreas, gallbladder and ovary. This study also demonstrated expression in day 1 embryos (Takeda and Suzuki, 1999). Northern blot analysis, which is slightly less sensitive than RNAse protection assays, only detected NPR-B mRNA expression in the rectal gland and kidney of *S. acanthias*, but it is worth noting that the gill was not assayed (Aller et al., 1999). NPR-C expression was demonstrated in the majority of tissues examined from eel, with significant expression in the gills, heart, brain and posterior intestine. Most tissues indicated a downregulation when fish were transferred from FW to SW (Takashima et al., 1995). RNAse protection assay that measured NPR-D expression in various tissues of the eel showed greatest expression in the brain followed by the gill and liver, with some expression elsewhere. There was no examination of the expression in SW eels, so whether there is an effect of salinity is unknown (Kashiwagi et al., 1995). In summary, it appears that using sensitive techniques for demonstrating mRNA expression of the receptors, the majority of tissues express them. The gill, digestive tract and accessory organs, brain, kidney, reproductive organs and heart seem to be the main sites of expression, with subtle differences between the receptor types.

Although mRNA expression is limited to those species for which the cDNA sequence for the receptors is known, competitive ligand binding studies using iodinated peptides in isolated membrane preparations offer another way of examining the general expression of NPR proteins in the tissues of various species (Table 14.1). Studies in the eel have used the native peptide that has been iodinated (e.g., Sakaguchi et al., 1993, 1996; Mishina and Takei 1997), but other studies have relied on iodinated mammalian peptides, and have demonstrated binding sites in all classes of fishes (e.g., Broadhead et al., 1992; Cerra et al., 1992, 1997; Donald et al., 1994, 1997; Toop et al., 1995a,b, 1998; Vallarino et al., 1996; Callahan et al, 2000). In general, these studies have focussed on major tissues involved in cardiovascular or osmoregulatory control. Because studies using radioligands cannot distinguish between the GC receptors and NPR-C, attempts have been made to quantify the proportion of GC receptors to NPR-C using the competition of C-ANF for specific radiolabeled NP binding sites. Using this method, the majority of binding sites appear to be of the NPR-C type (see, for example, Donald et al., 1994;

Mishina and Takei, 1997; Sakaguchi and Takei, 1998; Fig. 14.7). The prevalence of NPR-C type receptors in gill tissue is given credence by the study of Olson and Duff (1993), which demonstrated that 60% of circulating iodinated rat ANP was cleared from the circulation by the gills. This clearance was significantly diminished when SC 46542 (a mammalian NPR-C blocker) was injected before and in combination with the labelled peptide. Binding studies in the gill have generated calculated dissociation constants in the range of 1 – 100 nM, and β_{max} values between 50 and 500 fmol.mg protein^{-1} (e.g., Donald et al., 1994, 1997; Toop et al., 1995a; Mishina and Takei 1997; Sakaguchi and Takei 1998).

One of the advantages of using a radiolabelled peptide is that autoradiography of tissue sections can pinpoint receptors to cell type (Table 14.1). In the gills, binding has been located to a variety of tissue types, depending on species. In gulf toadfish, *Opsanus beta*, binding was isolated predominantly to vascular tissue and not to epithelial tissue. Binding was observed mainly on efferent blood vessels, although there was some binding to afferent vasculature (Fig. 14.7; Donald et al., 1994). In two species of Antarctic icefish, *Chiondraco hamatus* and *Pathogenia bernacchii*, specific binding was demonstrated on chloride cells (Uva et al., 1993), and Broadhead et al. (1992) suggested that the binding observed in dispersed cell preparations from the gills of European eel, *Anguilla anguilla,* was to chloride cells, since binding was greatest in SW eel cells. In the Japanese eel, specific binding was observed on parenchymal cells, which include chloride cells, and therefore the suggestion of Broadhead et al. (1992) is supported (Sakaguchi et al., 1993). In the same study, Sakaguchi and co-workers demonstrated an intense specific binding on the chondrocytes of gill cartilage. The majority of receptors in all these studies are believed to be of the NPR-C type. In dogfish, *S. acanthias*, mainly NPR-C binding sites occurred on the secondary lamellae and filament epithelium (Donald et al., 1997). In the gills of Agnatha, binding sites are localized to the lamellar folds of the gills pouches and not on the vasculature of two species, *Myxine glutinosa* and *E. cirrhatus* (Toop et al., 1995a; Callahan et al., 2000). In the pouched lamprey, *G. australis*, specific binding occurred on the endothelial tissue of the secondary lamellae, including pillar cells, and in the marginal channels, but not on epithelial tissue (Toop et al., 1998). In other tissues, specific binding has been localised to the vascular endothelium (Kloas et al., 1988; Toop et al., 1995b, 1998), to the renal glomerulus in the hagfish, *M. glutinosa,* and teleosts (Kloas et al., 1988; Uva et al., 1993; Toop et al., 1995b; Sakaguchi

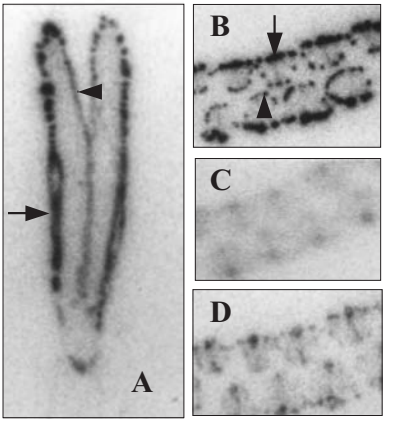

Fig. 14.7 Autoradiographs of the gill from toadfish, *Opsanus beta*, showing ^{125}I-rat ANP binding sites in the gill filaments. A is a transverse section to the gill arch, and B, C, and D are transverse sections to the gill filament. A and B show that the ^{125}I-rat ANP binding is confined to the afferent (arrowheads) and efferent (arrows) edges of the gill filaments (arrows), and microautoradiography showed that binding was to blood vessels (not shown). C and D are adjacent sections to B and show non-specific binding (C) after incubation of the sections with ^{125}I-rat ANP and excess unlabelled rat ANP, and residual binding (D) after incubation of the sections with ^{125}I-rat ANP and excess unlabelled C-ANF, a specific ligand for NPR-C. It can be clearly seen that the majority of ^{125}I-rat ANP binding is to NPR-C, but some residual binding is present, presumably to GC receptors. Modified from Donald et al. (1994).

et al., 1996), and to the apical and or basolateral membranes of kidney tubules (Uva et al., 1993; Toop et al., 1995b). The heart is also a target organ for NPs and studies have located cardiac binding sites in teleosts and dogfish (Cerra et al., 1992, 1997). In adrenal tissue of the teleost, *Cyprinus carpio*, NP binding sites were found throughout, suggesting that binding sites are present on both steroidogenic and catecholaminergic chromaffin cells (Kloas et al., 1994).

Using homologous antibodies to eel NPR-A and NPR-B and immunohistochemistry, Healy et al. (2005) examined the location of these receptors in the FW and SW eel kidney. NPR-B immunoreactivity was located on the smooth muscle of blood vessels, including that of the afferent and efferent arterioles, and also on smooth muscle surrounding the collecting ducts. NPR-A immunoreactivity, on the other hand, was located on the vascular endothelium, including the glomerular capillaries, as well as on the apical membrane of the proximal tubule 1 epithelium. There was no difference in intensity of signal or in distribution of the immunoreactivity between FW and SW eels. It is interesting that the only

transport epithelium showing NPR immunoreactivity is proximal tubule 1; this segment is apparently important for the sodium-coupled reabsorption of glucose and other macromolecules, and is functionally similar to the proximal tubule of tetrapods. The results from this study indicate that NPR-A and NPR-B are distributed differentially in fishes and future studies using these antibodies will undoubtedly provide additional information on the distribution of the GC receptors.

Interestingly, although the eel NPRs appear highly specific for the eel ligands, with little affinity for non-homologous peptides (Kashiwagi et al., 1999), studies in other fishes indicate that the stringency for NP binding is more relaxed (Sakaguchi and Takei, 1998; Smith et al., 2000; Callahan et al., 2002). These findings are significant when considering the new information that has become available through fish genome-sequencing projects. We must cautiously treat previous studies that considered only three ligands (ANP, CNP and VNP) and three receptors (NPR-A, NPR-B and NPR-C). We now know that the complement of NPs in bony fishes can be variable, with a number of CNPs, as well as ANP, VNP and BNP, being variably present in the genome of a particular species (Inoue et al., 2003a; Kawakoshi et al., 2004). It has been shown that the four medaka CNPs bind differentially to the medaka NPR-B homologue (OlGC1) and the two NPR-A homologues, OlGC2 and OlGC7. All four CNPs bound to the OlGC1 (NPR-B) but at different affinities, with CNP-4 showing the greatest activity, which is interesting since CNP-4 is the gene from which all tetrapod CNPs have evolved. Meanwhile OlGC2, an NPR-A homologue, was only activated by CNP-3, the ancestral peptide to ANP and BNP, and OlGC7, the other NPR-A homologue, showed greatest affinity for CNP-1 and CNP-2. Both CNP-1 and CNP-2 are exclusive to the bony fish lineage (Inoue et al., 2003a). We know nothing of the binding affinities of fish BNP for receptors. With the advent of this recently discovered complexity, at least to the Osteichthyan NP system, the study of NPRs has become the next major challenge and undoubtedly some of the older studies will need to be reviewed in light of recent research.

Another method of defining NP activity has been to use the peptides to stimulate cGMP formation in specific tissues. Measurement of cGMP in whole tissues, cells or cell membranes following stimulation with NPs is a reliable way of assessing the importance of either NPR-A or NPR-B in a particular tissue type. Although heterologous peptides have been used in numerous studies, the significance of the use of a homologous system depends on the promiscuity of specific receptors (see, for example,

Callahan et al., 2002). In Agnatha, the gills and kidneys are stimulated by both ANP and CNP to produce cGMP in a dose-dependent manner (Toop, 1995a,b; Toop et al., 1998; Callahan et al., 2000). In sharks, CNP stimulates cGMP production in the kidneys, rectal gland, interrenals, gills, intestine and brain (Donald et al., 1997; Gunning et al., 1997; Sakaguchi and Takei, 1998). Finally, in teleosts, NPs activate GC receptors in head kidneys, intestine, swimbladder, urinary bladder, gills, kidneys and brain of teleosts (Broadhead et al., 1992; Donald et al., 1994; Mishina and Takei, 1997; Callahan et al., 2002). In teleosts, CNP is the major stimulator of cGMP in gills, while ANP appears more effective in the kidney (Callahan et al., 2002).

Receptor studies have provided an indication of the target tissues for NPs. In many of these tissues, we are still unclear of the exact function of the NP system. Autoradiographic studies have indicated non-vascular binding in certain tissues (e.g., gills, kidneys, adrenals). However, since all tissues have an adequate blood supply, the vasculature is likely to express receptors and could confound the interpretation of results in methodologies that utilize whole tissues, for example, mRNA expression studies.

CARDIOVASCULAR REGULATION

The main site of synthesis and secretion of ANP and VNP in teleost fishes, and CNP in sharks and eels, is the heart (Tervonen et al., 1998; Takei et al., 2001). In keeping with their roles as cardiovascular modulators, the stimuli for release of NPs appear to be associated with an increased demand on the heart (Farrell and Olson, 2000) (Table 14.1). In addition, it appears that increased plasma osmolarity is a potent stimulus for NP release from eel cardiac myocytes (Kaiya and Takei; 1996). Some of the earliest experiments have demonstrated that isolated blood vessels respond to NPs by dilating, in either preconstricted or non-preconstricted preparations, in all classes of fishes (see Loretz and Pollina, 2000 for review). While the majority of these studies used mammalian peptides, the vasorelaxant response has been demonstrated in the killifish, *F. heteroclitus*, salmon, *S. salar*, trout, and eel using homologous peptides (Takei et al., 1989, 1997; Price et al., 1990; Olson et al., 1997; Tervonen et al., 1998; Evans and Harrie, 2001; Inoue et al., 2003b).

The overall effects of NPs on blood pressure are more difficult to interpret, possibly because of the range of peptides used, and routes and concentrations of administration. Comprehensive examination of the

cardiovascular effects in the trout, using homologous VNP and rat ANP, have demonstrated a rapid and sustained hypotensive effect of both ANP and VNP infused at a rate of 4.5 nmol.kg^{-1}.h^{-1} (Olson et al., 1997). This study demonstrated a rapid decrease in branchial resistance, central venous pressure, cardiac output and stroke volume, while paradoxically systemic resistance and heart rate increased. The interpretation of these data is that the major hypotensive effect of NPs in trout is via a decrease in venous compliance, reducing venous return, and consequently cardiac output (Olson et al., 1997; Farrell and Olson, 2000). However, there are studies using homologous peptides that have failed to show a change in mean arterial pressure. Neither eel ANP infused at a dosage of 0.3-3.0 pmol. kg^{-1}.min^{-1} into FW and SW eels, nor a bolus injection of sCP at 60 pmol.kg^{-1} into salmon altered blood pressure (Takei and Kaiya, 1998; Tervonen et al., 2002). The latter study, because of the diuretic effect of sCP (discussed below), concluded that the system would protect against volume overload, but not short-term perturbations of blood pressure. Another study in North American eels, *Anguilla rostrata,* demonstrated that eel ANP injected into a caudal vein catheter at a concentration of 150 ng.kg^{-1} reduced cardiac output and dorsal aortic pressure, but not systemic resistance (Oudit and Butler, 1995). In an early trout study, a bolus injection of human ANP failed to alter blood pressure, but did reduce pulse pressure by 60% (Eddy et al., 1990). Some studies have indicated that the administration of homologous or heterologous peptides leads to a biphasic pressor/depressor response. A very early study that administered rat ANP (0.1 μg.kg^{-1}) in trout demonstrated the pressor effect (Duff and Olson, 1986). A later study again demonstrated a pressor/depressor response with a bolus injection of rat ANP, but continuous infusion of rat ANP (300 ng.kg^{-1}.min^{-1}) only decreased the mean arterial pressure and pulse pressure and increased heart rate (Olson and Duff, 1992). If the α-adrenoreceptors were blocked with phenoxybenzamine, the pressor response to bolus ANP administration was prevented, and a transient hypotension was unmasked. This finding led to the hypothesis that either circulating catecholamines or adrenergic neural activity was responsible for the initial pressor response (Olson and Duff, 1992). Similar vasopressor/depressor responses have also been demonstrated using bolus trout VNP (and human ANP) administration, and the pressor responses were again eliminated by pretreatment with phenoxybenzamine (Takei et al., 1994b). McKendry et al. (1999) investigated the mechanism of the pressor response in several species. In the eel, *A. rostrata*, bolus injections

(1 nmol.kg^{-1}) of eel ANP or VNP decreased arterial blood pressure, but bolus injections (1 nmol.kg^{-1}) of rat ANP or trout VNP in trout demonstrated the usual pressor/depressor response. Measurements of circulating catecholamines were unaltered during this time. In addition, a posterior cardinal vein preparation perfused with NPs failed to elicit an increase in catecholamine release. CNP administered in the same way to the dogfish, *S. acanthias,* produced a pressor/depressor response with an attendant increase in circulating noradrenaline, indicating that the pressor response is probably mediated via the humoral system in sharks but via the sympathetic system in trout. However, a later study in dogfish was unable to demonstrate a direct connection between CNP perfusion and catecholamine release from chromaffin cells (Montpetit et al., 2001).

Interestingly, in isolated gill pouches of the hagfish, *E. cirrhatus*, perfused with increasing concentrations of mammalian ANP or CNP, a pressor/depressor effect on afferent branchial perfusion pressures was recorded at low concentrations (10^{-14}-10^{-10} M), but only depressor responses were observed at higher concentrations (Simpson et al., 2001). In addition, at low concentrations, ANP increased tension in isolated afferent branchial rings and was a relaxant at higher concentrations. In the absence of an intact system, the authors conclude that the increase in vascular tension is unlikely to be due to catecholamines. It is worth noting that higher concentrations of peptides switched branchial flow from the arterial to the venous route, which would direct blood to the peribranchial sinus. Such a mechanism may serve to protect the heart by reducing the volume of centrally circulating blood (Simpson et al., 2001). A reduction in the branchial resistance of teleosts has also been observed in older studies using heterologous peptides (Evans et al., 1989; Olson and Meisheri, 1989). Presumably, a reduction in branchial resistance is one mechanism by which afterload on the heart can be reduced, as discussed by Farrell and Olson (2000).

Renal effects of NPs can be considered both in terms of blood pressure regulation, through their ability to control blood volume, or osmoregulation, in their potential ionoregulative role. Since the kidneys of fishes are generally considered to be volume-regulating organs rather than a primary site of control of NaCl, we will consider the role of NPs in the kidney under the auspices of cardiovascular regulation, while bearing in mind that alterations to fluid volume can impact on the osmoregulation of fishes (Table 14.1). Studies in teleosts have shown that mammalian peptides are diuretic in trout, probably by an increase in glomerular

filtration rate (Duff and Olson, 1986; Olson and Duff, 1992; Duff et al., 1997). In an earlier study (Duff and Olson, 1986), the excretion of K^+, Na^+ and Cl^- was also increased. Olson and Duff (1992) have also demonstrated that eel ANP is diuretic in trout. In salmon, a bolus injection of sCP (60 $pmol.kg^{-1}$) was diuretic and natriuretic, with the natriuresis being proportional to the increase in urine flow rate (Tervonen et al., 2002). In contrast, when infused at doses that did not alter blood pressure, eel ANP has been demonstrated to be antidiuretic in SW eels (Takei and Kaiya, 1998). There was no change observed in the amount of NaCl excreted, although due to the reduction in urine volume, urinary NaCl concentration increased. An earlier study demonstrated that eel ANP and VNP produced antidiuresis in FW eels, but again, without significant changes in Na^+ excretion (Takei and Balment, 1993). ANP has also been shown to be antidiuretic and antinatriuretic in the dogfish, *S. acanthias,* but since CNP is the only NP in sharks, these data are difficult to interpret (Benyajati and Yokota, 1990). The aglomerular toadfish, *Opsanus tau*, experiences natriuresis and diuresis when a bolus injection of either homologous heart extract or synthetic ANP are administered, indicating that these responses may be achieved through tubular secretion and not merely glomerular filtration (Lee and Malvin, 1987). The natriuresis is interpreted as being due to an inhibition of Na^+ reabsorption rather than an increase in Na^+ secretion. Interestingly, there were no changes in mean arterial pressure with ANP injection, although heart extract did induce a transient decrease, perhaps indicating dissociation between hypotensive and renal effects. Duff et al. (1997) demonstrated that the urine flow rate could only account for 40% of the observed decrease in extracellular fluid volume, and concluded that ANP could influence the translocation of fluid into other body compartments, including the intracellular compartment. The combined effect of decreased blood volume (determined by both urine flow and translocation of fluid) and the previously discussed increase in venous capacitance (Olson et al., 1997) would have the net effect of decreasing venous return and thus reducing cardiac output and, therefore, load on the heart.

In general, the observed hypotensive effects would appear to be the result of combined effects on the vasculature and extracellular fluid volume, although not all studies support a role in systemic blood pressure reduction. Nevertheless, there is considerable support for Farrell and Olson's hypothesis that a primary function of the NP system in fishes is cardioprotection (Farrell and Olson, 2000).

EPITHELIAL REGULATION

Intestine

A critical aspect of fish osmoregulation is not only the transport of ions but also the uptake and excretion of water, depending on environmental salinity. Apart from osmosis of water across permeable body surfaces, water can be acquired across the intestine by drinking. Subsequent to the arrival of water in the intestine, water moves into the body following the transepithelial passage of salt. This latter mechanism is particularly important for osteichthyan fishes in a saltwater environment because their internal osmolality is less than that of the surrounding media. As a consequence, they lose water passively to the environment and must offset this dehydrational loss by drinking (Takei, 2000b). Because of the relationship between drinking and the intestinal transport of salt, we are considering the effects of NPs on both these parameters together.

Early studies indicated that ANP and VNP inhibited the drinking rate in both FW and SW eels (Takei and Balment, 1993). A later, in-depth study (Tsuchida and Takei, 1998) examined the relationship between eel ANP, angiotensin II and drinking in SW, FW and FW hemorrhaged eels, by infusing increasing doses of ANP (0.3, 1.0 and 3.0 $pmol.kg^{-1}.min^{-1}$) over a two-hour period. Drinking rate and plasma ANP and angiotensin II concentrations were measured. ANP was shown to be antidipsogenic in all groups of eels and the drinking inhibition was accompanied by a reduction in plasma angiotensin II. It is unclear whether the antidipsogenic effect of ANP was a secondary effect of the reduction of plasma angiotensin II, which is dipsogenic, or due to direct effects of ANP on the brain (see below). Interestingly, a bolus injection of CNP alone had no effect on drinking rate in the dogfish, *S. canicula*; however, angiotenisn II-stimulated drinking was significantly reduced when a combination bolus injection of both angiotensin II and CNP was administered. This suggests that CNP may have a role in inhibiting angiotensin-stimulated drinking in chondrichthyans (Anderson et al., 2001).

In addition to inhibiting drinking, many studies have consistently demonstrated that NPs inhibit sodium chloride transport across the teleost intestine and this effect is mimicked by the cGMP analogue 8-bromo-cGMP, which suggests that the inhibition is via a GC receptor (O'Grady et al., 1985; Ando et al., 1992; Ando and Hara, 1994; Loretz, 1995; Loretz and Takei; 1997) (Table 14.1). Using homologous eel peptides, the order of potency for the inhibition of net epithelial salt

absorption, as measured by the inhibition of short circuit current, was amidated eel ANP > VNP > ANP >>CNP, in both FW and SW eels. The order of potencies clearly suggests that the inhibition is mediated via the NPR-A (Loretz and Takei, 1997). Interestingly, this study showed that the effective concentration of ANP and VNP required to inhibit salt transport across the intestine is an order of magnitude greater than the circulating concentrations in the plasma. Further investigations demonstrated that the intestine synthesizes ANP and VNP locally throughout the intestinal epithelium, pointing to paracrine actions of ANP and VNP in the gut (Loretz et al., 1997). Loretz and Takei (1997) propose that the NP-activated inhibition of salt absorption across the intestine may be for purposes of localized nutrient absorption rather than for osmoregulation. Since nutrient absorption is coupled with Na^+ transport, the inhibition of NaCl and Na^+-K^+-Cl coupled transport would favour the use of Na^+ in nutrient absorption when necessary. Since little difference was observed with either HPLC measurement of NPs or specific NP immunoreactivity in the gut of FW and SW eels, this hypothesis is given further credence as the need to absorb nutrients is present regardless of salinity. In addition, RNAse protection assays indicated little difference in the expression of NPR-A mRNA in the intestines of FW- and SW-adapted eels (Kashiwagi et al., 1999), although a ligand-binding study demonstrated greater binding of iodinated eel ANP in the anterior intestine of FW- acclimated as opposed to SW-acclimated eels (Mishina and Takei, 1997).

When the dual effects of ANP are considered, i.e., the transient decrease in drinking rate in SW eels and the inhibition of intestinal salt absorption, an alternative osmoregulatory model is proposed. The initial increase in plasma ANP on transfer of eels to SW (Kaiya and Takei, 1996) transiently inhibits drinking, which together with the (potentially locally-mediated) inhibition of intestinal salt uptake, protects the fish from excessive salt load immediately following transfer of fish to SW, thus promoting SW adaption (Takei and Hirose, 2002).

Rectal Gland

Unlike osteichthyan fishes that rely on branchial sites for NaCl extrusion in SW osmoregulatory control, chondrichthyans possess a specialized organ for this function, the rectal gland, that uses similar transport mechanisms to accomplish NaCl secretion as the mitochondrial-rich cells of the gills of SW teleosts (Valentich et al., 1995; Karnaky, 1998). The

majority of research into NP function in this group has concentrated on rectal gland regulation (Table 14.1). Very early studies demonstrated that a bolus administration (10 $\mu g.kg^{-1}$) of rat ANP stimulated Cl^- secretion from *in vivo* perfused rectal glands of *S. acanthias* (Solomon et al., 1985). This secretion was later shown to be the result of ANP stimulating vasoactive intestinal peptide (VIP) release from nerves (Silva et al., 1987), although a direct effect was indicated by Karnaky et al. (1991) when a variety of mammalian forms of ANP stimulated both cGMP formation and Cl^- release from cultured rectal gland cells. Following the discovery that CNP appeared to be the cardiac NP in sharks (e.g., Schofield et al., 1991; Suzuki et al., 1991, 1992), rectal gland research focused on CNP. CNP was shown to be a potent stimulator of rectal gland Cl^- secretion and a more potent stimulator of cGMP formation than either ANP or BNP (Solomon et al., 1992; Gunning et al., 1993). A detailed discussion of early work on the role of NPs in rectal gland function can be found in Valentich et al. (1995). Several more recent studies indicate that the effects of CNP on *S. acanthias* rectal gland Cl^- secretion are highly complex and involve more than a single mechanism. Cardiac CNP, which is released on volume expansion, stimulates the NPR-B, and via cGMP formation, stimulates the secretion of Cl^- (Gunning et al., 1997). *Xenopus* oocytes expressing both the *S. acanthias* NPR-B and the CFTR Cl^- channel were stimulated by CNP to produce cGMP and to secrete Cl^- (Aller et al., 1999). Furthermore, CNP stimulates neural VIP release from the rectal gland, which stimulates cAMP and Cl^- secretion, as well as stimulating Cl^- secretion directly in rectal glands in which VIP release is blocked (Silva et al., 1999). The actions of CNP appear to be mediated through the production of cGMP by the NPR-B and by activation of protein kinase C, possibly by another receptor, and the effects of these systems are synergistic (Silva et al., 1999). Apart from direct and indirect effects on Cl^- transport, CNP increases the perfusion of rectal gland epithelia (Anderson et al., 2002) and relaxes the circular smooth muscle band that surrounds the periphery of the rectal gland (Evans and Piermarini, 2001). These changes could indirectly alter the Cl^- secretion rate.

Gills

The gills have long been recognized as a major target for cardiac NPs. Although NPs dilate the ventral aorta (e.g., Evans et al., 1989, 1993; Benyajati and Yokota, 1990; Evans and Harrie, 2001), the gills contain the first major concentration of NPRs encountered by NPs secreted from the heart. We have already discussed the branchial receptors above. While it

is accepted that NPs alter branchial resistance, there are surprisingly few studies showing a direct effect of NPs on ion transport across the gill epithelia (Table 14.1). In teleosts, early studies determined that heterologous ANP stimulated chloride secretion (short circuit current) across an Ussing chamber opercular membrane preparation from FW and SW killifish (Scheide and Zadunaisky, 1988). The effect was dose-dependent and only observed when ANP was applied to the serosal side of the membrane. A more recent study (Evans, 2002) failed to elicit the same effect using eel ANP and porcine CNP, therefore, it is difficult to interpret these results. Whole animal studies indicate that Na^+ efflux increases in SW fishes treated with ANP (Arnold-Reed et al., 1991). In eels, eel ANP and eel VNP decrease plasma Na^+ concentrations in SW but not FW animals (Takei and Kaiya, 1998; Tsukada and Takei, 2001). Since the profound decrease in plasma Na^+ could not be accounted for by the renal route alone, it was believed that the gills were a likely site of transport (Takei and Kaiya, 1998; Takei and Hirose, 2002). However, a recent study showed that plasma Na^+ concentration in SW eels is reduced via the inhibition of Na^+ uptake across the intestine with no increase in branchial secretion (Tsukada and Takei, pers. comm.). In this study, ^{22}Na fluxes across the whole body were unaltered by ANP infusion, although drinking rate and intestinal uptake of Na^+ were inhibited.

While ANP is able to stimulate cGMP formation in the gill, studies have demonstrated that CNP stimulates cGMP to a greater extent in this tissue (Mishina and Takei, 1997; Callahan et al., 2002). The effectiveness of CNP in stimulating GC activity in the gills is probably due to its ability to bind to both NPR-B and NPR-A receptors. Interestingly, Inoue et al. (2003) have demonstrated that the four teleost CNPs have differential abilities to activate NPR-A homologues in medaka. Any effects of NPs on the gill must also be considered in the light of the recent study demonstrating a probable involvement of the NPR-C receptors in cAMP signaling in the gills, although these are down-regulated in sea water (Takashima et al., 1995; Callahan et al., 2004; Fig. 14.6). It also is interesting that the gills, along with the brain, are the site of NPR-D expression, the function of which remains to be elucidated (Kashiwagi et al., 1995).

ENDOCRINE INTERACTIONS

It is not surprising that NPs have been shown to interact with other effectors of cardiovascular and osmoregulation and we examine some of this information here. Interactions between NPs and the renin

angiotensin system, cortisol, catecholamines, growth hormone and prolactin have been examined. We have already discussed the effect of ANP on angiotensin II and drinking rate in eels (Tsuchida and Takei, 1998; Anderson et al., 2001), and the effect of CNP on VIP release from nerves in the rectal gland (Silva et al., 1999), above.

Cortisol is produced by the interrenal cells of fishes and is released into the bloodstream of both FW and SW fishes. It is believed to act in synergy with growth hormone to stimulate the activity of gill Na^+,K^+ ATPase in SW fishes (McCormick, 2001). Some earlier studies, using mammalian NPs, have demonstrated a link between the cortisol system and the NP system (Table 14.1). Infused human ANP increased circulating cortisol concentrations in SW-acclimated flounder and rainbow trout gradually acclimated to sea water; however, FW trout showed little cortisol response to ANP infusion (Arnold-Reed and Balment, 1991). Similar results were obtained in SW, but not FW eels, when eel ANP was injected. Eel VNP was without effect (Takei and Balment, 1993). Cortisol secretion was observed to increase, on the other hand, in rat ANP-perfused interrenal cells of the freshwater carp (Kloas et al., 1994). NPR-A, and (to a lesser extent) NPR-B mRNA expression, have been observed in the interrenal tissue of the Japanese eel (Katafuchi et al., 1994; Kashiwagi et al., 1999). A recent study has demonstrated that eel ANP increased circulating cortisol in SW eels, but only CNP did so in FW animals (Li and Takei, 2003). Finally, cortisol may be linked in a positive feedback fashion to enhance cardiac secretion of NPs in rainbow trout. Powell and Miller III (1992) showed that dexamethasone (a synthetic cortisol analogue) stimulated the release of ANP from ventricular cells.

In addition to cortisol production, certain pituitary hormones are involved in the osmoregulation of fishes. Prolactin is important in maintaining ion and water permeability in osmoregulatory organs of FW fishes, while plasma levels of growth hormone increase on acclimation to seawater. In concert with cortisol, growth hormone enhances Na^+,K^+ ATPase activity in SW gills (Hazon and Balment, 1998). Eckert et al. (2003) have recently demonstrated in the isolated pituitary of *O. mossambicus* that none of the eel NPs affected secretion of prolactin, while growth hormone secretion was significantly elevated between 4 and 48 hours subsequent to VNP administration. This elevation was dose dependent at concentrations above 1nM. CNP also stimulated growth hormone release, but less effectively than VNP, and ANP failed to elicit

a response. This result is interesting because it is one of the few occasions when VNP is more potent than either ANP or CNP.

Several studies have looked at the potential link between catecholamine secretion and the NP system, particularly as ANP can inhibit catecholamine release from the adrenal glands in mammals (cited in Reid et al., 1998). Catecholamines have several physiological functions in fishes, mostly directed towards maintaining oxygen supply to tissues and meeting metabolic needs in a variety of situations. Included in these functions are vasoconstriction, and branchial vasodilation and ion-regulation (Hazon and Balment, 1998). Binding sites for rat ANP have been observed in the head kidney of carp, and there is immunohistochemical evidence for ANP expression in adrenaline-synthesizing cells of the same tissue (Kloas et al., 1994). In the same study, rat ANP stimulated cortisol and acetylcholine-stimulated adrenalin secretion. However, a later study in eel and trout failed to show any regulation of catecholamine secretion following head kidney perfusion with mammalian ANP, eel ANP or eel VNP (McKendry et al., 1999).

PERSPECTIVE ON OSMOREGULATION AND NATRIURETIC PEPTIDES IN EELS

The most comprehensive data set for osmoregulatory function is for the eel (Takei and Hirose, 2002). Eels are very euryhaline and breed in the sea and migrate to freshwater for growth, and it is currently unknown how typical the eel NP system is of the role of NPs in osmoregulation generally. The current paradigm for the role of NPs in eel osmoregulation is that ANP is most important for short-term adaptation to seawater, while CNP is a FW-adapting hormone (Takei and Hirose, 2002). The roles of VNP and the recently discovered BNP in teleosts are not well understood for the former, and completely unknown for the latter. Presumably, like VNP, BNP can interact with all NPR subtypes. The role of CNP in FW osmoregulation is currently not well investigated because its potential significance in this role has only relatively recently been recognized (Takei et al., 2001). The fact that CNP circulates at high concentrations in FW eels rather than SW eels, and the elevated mRNA expression of NPR-B in FW eels, is significant in regard to its potential role (Takei et al., 2001). Nevertheless, CNP did not alter plasma Na^+ concentration, haematocrit or drinking rate in SW or FW animals (Tsukada and Takei, 2001).

In keeping with a role in SW osmoregulation, elevated plasma osmolarity increases cardiac ANP secretion (Kaiya and Takei, 1996). As

discussed above, ANP and VNP transiently inhibit drinking in SW eels, reduce circulating Na^+ levels (Tsuchida and Takei, 1998) and inhibit intestinal Na^+ uptake (e.g., Loretz and Takei, 1997). The inhibition of drinking and Na^+ uptake would seem to be contraindicated for SW fish that need to absorb Na^+ across the intestine so that water can be taken up. Tsuchida and Takei (2001) propose that the transient inhibition of drinking and the inhibition of intestinal Na^+ uptake protect the fish from extreme hypernatriemia on the initial entrance into SW. The transient increase in plasma ANP is believed responsible for these inhibitions (Takei and Hirose, 2002). The increase in circulating ANP also stimulates the release of cortisol (Li and Takei, 2003) and potentially growth hormone, since this has been shown in *O. mossambicus* (Eckert et al., 2003), and these are longer-acting osmoregulatory hormones (Takei and Hirose, 2002). The question of whether ion regulation across the gills is mediated directly or indirectly by NPs is interesting and has been discussed above. The renal actions of NPs in eels indicate that ANP infusion decreases urine flow, but increases urine Na^+ concentration but not Na^+ excretion (Takei and Kaiya, 1998), in keeping with a role in conserving water and offloading salt in a SW environment.

NATRIURETIC PEPTIDE SYSTEMS IN THE FISH BRAIN

Natriuretic peptides and their receptors are found in the brain of cyclostome, chondrichthyan, and bony fishes. However, apart from the structural data, there is very little information on the function of NPs in the brain. Interestingly, there is considerable homology in the distribution of NPs and NPR between fishes and higher vertebrates, which may permit some assessment of NP function.

Types and Distribution of Natriuretic Peptides and Receptors in the Brain

In the hagfish, *E. burgeri*, the NP cloned from the heart called EbuNP is also expressed in the brain (Kawakoshi et al., 2003). However, mass spectrometry analysis of brain samples affinity purified with EbuNP antiserum could not identify similar peptides to those observed in heart and plasma samples, and it was concluded that brain EbuNP is processed differently to cardiac EbuNP. Of considerable interest is the finding that *E. burgeri* is the first vertebrate species in which CNP has not been cloned from the brain. However, Kawakoshi et al. (2003) argue that brain EbuNP may be processed at the arginine residue following the second cysteine of

the intramolecular ring, which would give rise to a CNP-like peptide without a C-terminal tail. This may also explain the lack of detection of brain EbuNP by mass spectrometry because the EbuNP antibody could be directed to the long tail sequence due to its high immunogenicity.

Prior to the discovery of EbuNP in hagfish, immunohistochemistry had been performed using antibodies to mammalian NPs. Interestingly, immunoreactive neurons and axons were observed in the brain of the Atlantic hagfish, *M. glutinosa*, only when an antiserum was used that cross-reacted with CNP, and it is probable that this antibody is revealing the distribution of EbuNP in the brain. NP-immunoreactivity was found in the pallium, primordium hippocampi, the nucleus profundus, the nucleus tuberculi posteriosis, and the nucleus ventralis tegmenti (Donald et al., 1992). In an earlier study, Reinecke et al. (1987) found a more limited distribution of NP immunoreactive structures in the brain of *M. glutinosa* using an antibody to mammalian ANP. To date, the types of NPR in the cyclostome brain are unknown, but the distribution of ANP and CNP binding sites has been determined in the brain of *M. glutinosa*; interestingly, distinct binding patterns were observed for each ligand. Both ANP and CNP binding sites were observed in the olfactory bulb, the pallium of the telencephalon, the thalamus and the hypothalamus, but only ANP binding was found in the mesencephalon and the medulla (Donald et al., 1999).

In chondrichthyan fishes, CNP appears to be the only NP present in the brain, as determined by molecular cloning and peptide biochemistry. Suzuki et al. (1992) isolated CNP-22 from the brain of *T. scyllium*, but found that the heart contained proCNP-115, which again suggests that NP processing in the brain is different from the periphery. Subsequently, a CNP cDNA was cloned from the brain of *T. scyllium* that was identical at the nucleotide level to that cloned from the heart (Kawakoshi et al., 2001). Kawakoshi et al. (2001) then performed an experiment in which the CNP mRNA was removed from the mRNA pool prior to reverse transcription. Subsequent PCR using primers that targeted the conserved regions of the intramolecular ring of NPs failed to amplify any NPs, other than truncated CNPs. Thus, it was concluded that CNP is the only NP in the brain of *T. scyllium*. The expression of NPR has only been determined in one study in which NPR-B mRNA could not be detected in the brain of *S. acanthias*, using northern blotting (Aller et al., 1999); it is probable that a more sensitive method such as PCR would detect NPR-B transcripts in the brain.

Two immunohistochemical studies using heterologous antibodies to mammalian NPs have been used to study the distribution of NP-immunoreactive elements in the brain of *S. canicula* and *S. acanthias*. In *S. canicula*, immunohistochemistry was performed using an antibody to mammalian ANP and the bulk of the ANP-like immunoreactivity was found in the telencephalon and diencephalon, with only a few fibres being observed in the mesencephalon (Vallarino et al., 1990). In contrast, the study in *S. acanthias* was performed with an antibody known to cross-react with CNP, and interestingly, a more extensive distribution of NP-immunoreactive structures was found. In addition to the telencephalon and diencephalon, NP immunoreactivity was observed in the olfactory bulb, the tectum mesencephali, rhombencephalon, and spinal cord (Donald et al., 1992). Notably, in both the species, extensive immunoreactivity was observed in the preoptic region, tractus preoptico-hypophyseus, and intermediate lobe of the pituitary gland, which provides evidence that NPs in the brain are present in the hypothalamo-hypophyseal axis and may be involved in the regulation of pituitary function. Unfortunately, there are no data on the distribution of NP binding sites in the brain of chondrichthyans, which would complement knowledge of the location of NPs.

In bony fishes, CNP has been isolated from the brain of *F. heteroclitus* (Price et al., 1990) and *A. japonica* (Takei et al., 1990). Inoue et al. (2003a) have demonstrated that CNP-1, CNP-2, CNP-3 and CNP-4 are expressed in the CNS of medaka; in fact, CNP-1 and CNP-2 are exclusively expressed in the CNS. In addition, CNP cDNAs have been cloned from the brain of *A. japonica* (Takei et al., 2001), *A. transmontanus* (Kawakoshi et al., 2004), and two CNP cDNAs were cloned from the brain of rainbow trout, but the CNP-22 sequence was identical (Inoue et al., 2003b); both eel and trout CNP demonstrate closest similarity to CNP-1 (Inoue et al., 2003a). The expression of CNP mRNA in the brain is not different in eels adapted to fresh water or sea water, which is in contrast to peripheral tissues such as the heart, in which CNP expression is significantly enhanced in fresh water (Takei et al., 2001). However, in another study, the concentration of CNP in the brain of *O. tau* increased when fish were transferred from SW to 50% SW (Galli and Phillips, 1996). In addition to CNP, ANP is also expressed in the eel brain (Takei et al., 1997a), but the expression of VNP could not be detected with northern blotting (Takei et al., 1994b). In the brain of sturgeon, BNP mRNA is expressed at reasonable levels, but VNP mRNA is only weakly expressed, and no expression of ANP mRNA could be detected (Kawakoshi et al., 2004).

Prior to the discovery of the types of NPs in the teleost brain, immunohistochemistry using heterologous antibodies has shown NP-immunoreactivity in various brain regions of two teleost species. In the brain of the gulf toadfish, *O. beta*, immunohistochemistry using an antibody known to cross-react with CNP found immunoreactive perikarya in the preoptic region of the diencephalon, and many immunoreactive fibres in the telencephalon, preoptic area, and rostral hypothalamus, lateral to the thalamic region. Interestingly, no immunoreactivity was observed in the hypophysis. In addition, NP-immunoreactive fibres were observed in the thalamus, the dorsolateral regions of the midbrain tegmentum, and the tectum (Donald and Evans, 1992). In the brain of the Antarctic fish, *C. hamatus,* the use of antibodies to mammalian ANP showed NP immunoreactivity in the telencephalon, the periventricular hypothalamic region of the diencephalon, and the pars distalis of the pituitary, as well as more caudal brain regions. In the diencephalon, ANP immunoreactivity was observed in tanycytes that originate in the walls of the third ventricle (Pestarino et al., 2000). In addition to the two teleost species, an extensive analysis of NP-immunoreactive structures has been performed in the brain of the African lungfish, *Protopterus annectens*, using an antibody to mammalian ANP (Vallarino et al., 1996). NP-immunoreactivity was found in each brain region, particularly in the preoptic area of the diencephalon, and the pars intermedia and pars nervosa of the pituitary.

The eel brain expresses NPR-A (Kashiwagi et al., 1999), NPR-B (Katafuchi et al., 1994), NPR-C (Takashima et al., 1995), and the novel non-GC linked receptor NPR-D (Kashiwagi et al., 1995), but the specific distribution of each receptor in the brain is not known. NPR-D is a unique NPR that is primarily found in the eel brain, and is a tetrameric receptor that lacks a GC domain and binds the NPR-C specific ligand, C-ANF. In contrast to its ligand CNP, NPR-B expression is higher in the brain of freshwater rather SW eels (Katafuchi et al., 1994). In addition to eel, the NPR-A (OlGC7; Yamagami et al., 2001) and NPR-B (OlGC1; Takeda and Suzuki, 1999) homologues of medaka fish are expressed in the brain.

There are only two studies of the distribution of NP binding sites in the brain of bony fishes, namely *P. annectens* (Vallarino et al., 1996) and *C. hamatus* (Pestarino et al., 2000). Both studies used iodinated mammalian ANP as a ligand without the knowledge that ANP itself or NPR-A are expressed in the brain of the species being examined. This is

particularly relevant given that CNP is predominant NP in the brain of fishes. Furthermore, neither study discriminated between binding of ANP to NPR-A or NPR-C; thus, it is likely that most of the binding sites represent NPR-C, as is the case in most tissues of fishes. This does not rule out the possibility that ANP is signalling in the brain via NPR-C as has been shown in the gills of Australian short-finned eel (Callahan et al., 2004). In the brain of *C. hamatus*, a dense distribution of ANP binding sites was found in many regions of the telencephalon, diencephalon, mesencephalon, and in the whole pituitary gland (Pestarino et al., 2000). Similarly, in the brain *P. annectens*, ANP binding sites were broadly distributed in the brain, with particularly high levels of binding in the telencephalon, diencephalon and each lobe of the pituitary (Vallarino et al., 1996).

The Role of NPs in the Brain

The broad distribution of NPs and NPR in the brain of fishes indicates that NPs probably have a broad range of functions, but the nature of the functions can only be implied from the homologous distribution of peptides and receptors between the piscine and the mammalian brain. It is clear that NP systems are generally located in the hypothalamus, which implies that NPs may play an important central role in the maintenance of osmoregulatory and cardiovascular homeostasis. Furthermore, the presence of NPR on the pituitary shows that NPs are involved in hypophyseal secretory processes. For example, eel CNP and VNP provided long-lasting stimulation of growth hormone release from the pituitary of *O. mossambicus*, but had no effect on prolactin release (Eckert et al., 2003). However, it is important to emphasize that NPs and NPRs are widely distributed in the brain, particularly in the telencephalon, which suggests that the peptides are important neurotransmitter and/or neuromodulators in the central nervous system of fishes (see Vallarino et al., 1996; Pestarino et al., 2000).

Two recent research articles have further contributed to the knowledge of the structure, function and evolution of natriuretic peptides in fishes. Importantly, in a survey of bony fish (Inoue et al., 2005, see reference below), BNP, and not ANP, appears to be the peptide that is common to all species, since BNP alone was found in medaka. VNP has only been identified in sturgeon, eel and trout, and is absent from more 'advanced' teleosts. Linkage mapping in the rainbow trout indicates that

ANP, BNP and VNP are in the same position of the same linkage group, suggesting that VNP also originated from tandem duplication of ANP, BNP or CNP-3. In addition, medaka BNP stimulates cGMP formation in COS cells expressing the medaka NPR-A homologues, OIGC2 and OLGC7, which would make BNP, as well as ANP, a ligand for NPR-A Anderson et al., 2005, have published the sequence of CNP from the bull shark, *Carcharhinus leucas*. The mature peptide was identical in its amino acid sequence to that of two other elasmobranchs, *Triakis scyllia* and *Scyliorhinus canicula*. CNP mRNA expression decreased in the atrium in response to acclimation to SW but circulating concentrations of CNP increased.

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SECTION 5

Cardiac No Signaling

CHAPTER

15

Nitric Oxide Modulation of Mechanical Performance in the Teleost Heart

Bruno Tota*, Sandra Imbrogno and Alfonsina Gattuso

ABSTRACT

In mammals, nitric oxide (NO), produced by nitric oxide synthase (NOS) and released by myocardiocytes and/or endocardial and vascular endothelial cells, plays an important role in modulating both intrinsic and extrinsic cardiac regulatory mechanisms.

Fish hearts show common structural and functional aspects with higher vertebrates and, at the same time, differences in cardiac ultrastructure, myoarchitecture (trabecular versus compact type), blood supply (lacunary versus vascular) and pumping performance (sensitivity to filling pressure). They may thus represent useful experimental models to study an early cardiac autocrine-paracrine function of NO, possibly revealing the aspects of unity and diversity in the heart design.

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This synopsis focuses on the role of NO in modulating mechanical performance in teleost hearts. We show that NO, through a cGMP-mediated mechanism, regulates basal cardiac performance and influences the preload-induced increases in cardiac output at a constant afterload and heart rate (i.e., Frank-Starling relationship), an intrinsic mechanism crucial for cardiac physiology in fish. Moreover, NO is involved in modulating cardiac response to important chemical stimuli such as exogenous acetylcholine and angiotensin II. The involvement of cardiac NOS system in fish is also stressed by the role played by NO in the determinism of cardiac dysfunction associated with a viral disease as shown in salmon. Finally, the use of an avascular teleost heart, as a conceptual tool, illustrates the importance of a paracrine role of endocardial endothelium NO during the evolution of cardiovascular system in lower vertebrates.

Key Words: Cardiac performance; Teleosts; Nitric oxide; Endocardial endothelium.

INTRODUCTION: CARDIAC NO SIGNALING

The heart is able to adjust its performance in relation to the changing demands of the organism. This ability is attained through intrinsic (Frank-Starling mechanism, frequency/force relationship, shear stress, etc.) and extrinsic (neural and humoral) mechanisms. In mammals, all these mechanisms are modulated by nitric oxide (NO). NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). Three NOS isoforms (NOS1 or nNOS, NOS2 or iNOS and NOS3 or eNOS) have been identified in cardiomyocytes, being localized in key organelles allowing precise and spatially confined regulation of various cellular activities (Barouch et al., 2002). Generated and released by various cardiac tissues, e.g., myocardiocytes, endothelial (both vascular and endocardial) cells, interstitial cells, coronary vessels and myocardial neurons, NO orchestrates, in an autocrine-paracrine manner, a variety of cardiac activities (Balligand, 2000; Hare, 2003). For example, NO alters diastolic properties, modulates the Frank-Starling response, the force-frequency relationship, the myocyte contractility as well as the ß-adrenergic and cholinergic inotropic effects (Brutsaert, 2003; Khan et al., 2003, and references therein).

NO is also involved in the alteration of transduction pathways in cardiac myocytes during sepsis or after exposure to cytokines (Brutsaert, 2003; Massion and Balligand, 2003).

NO may modulate cardiac contractility through a cGMP-dependent mechanism. However, cGMP-independent mechanisms may operate through interaction of NO with heme proteins, non-heme iron, or free thiol residues on target signaling proteins, or ion channels (Balligand and Cannon, 1997; Hare, 2003).

The fish heart is a venous heart since it is perfused only by venous blood. In comparison with higher and warmblooded vertebrates, it is designed as a low-pressure region exposed to relatively low and variable levels of pO_2, being endowed with stretch sensors for changes in pressure and volume gradients of the venous return (Aardal and Helle, 1991; Farrell and Jones, 1992). Moreover, the myocardial and the endocardial endothelial (EE) cells of most teleost hearts are particularly rich in specific secretory granules that constitute the final step in the regulated secretory pathway for a number of cardiac hormones such as the atrial natriuretic peptides (Aardal and Helle, 1991). This stresses the importance of the fish heart as an endocrine-paracrine organ (Aardal and Helle, 1991). The teleost heart differs from the homeotherm heart in myoarchitecture (trabecular versus compact type), blood supply (lacunary versus vascular) (Tota et al., 1983), myocardial ultrastructure (lack of T-tubes) and pumping performance (e.g., sensitivity to filling pressure) (see for references Farrell and Jones, 1992; Tota and Gattuso, 1996). Taken together, these heart features challenge studies addressed on how NO signaling regulates cardiac function in fish. These studies may uncover aspects of unity and diversity in cardiac NO functions which are of evolutionary and adaptive significance and, at the same time, may also reveal both conserved and novel aspects of the pleiotropic role exerted by NO in the present-day mammalian heart. However, the role of NO in the control of cardiovascular function in fish has been, till now, scarcely studied and appears contentious because of contradictory results, which may be attributable to either different organ-tissue preparations used (as detailed below) and/or possible species-specificities (see for references Pellegrino et al., 2002).

Here, we will summarize the work from our laboratory, which has established the role of NO in various teleost hearts, outlining the influence of NO on basal mechanical performance, Frank-Starling relationships, chemical modulation and EE-dependent regulation of myocardial function.

We have deliberately used *in vitro* isolated and perfused whole heart preparations working at 'physiological' loading (preload and afterload) conditions. This kind of preparation allows the evaluation of cardiac performance free from extrinsic neuro-humoral stimuli, while the controlled nature of the perfusion fluid may reduce the removal rate of NO and the production of peroxynitrites (Voelkel et al., 1995). Furthermore, in a beating ejecting heart, 'silencing' eNOS, which may remain inactive in non-working (e.g., unloaded myocytes) cardiac preparations (Vila- Petroff et al., 2001; Wu, 2002), becomes activated, hence revealing the presence of 'physiological' NO basal tone.

NO AND BASAL MECHANICAL PERFORMANCE

In eel (*A. anguilla*; Imbrogno et al., 2001) and salmon (*S. salar*; Gattuso et al., 2002) hearts, under basal (i.e., unstimulated) conditions, a tonic release of NO exerts a mild negative inotropism thereby modulating mechanical performance. In fact, in these *in vitro* working hearts, stroke volume (SV) and stroke work (SW), used as important parameters of myocardial inotropism, were reduced by treatments with exogenous NO donors while were enhanced in the presence of NOS antagonists. In particular, in the eel heart this basal nitrergic tone involves a cGMP-dependent mechanism (Fig. 15.1).

In mammals, the inotropic action of increased NO levels has been reported to be negative (Meulemans et al., 1988; Balligand et al., 1993; Grocott-Mason et al., 1994; Paulus et al., 1994), positive (Kojda et al., 1996; Mohan et al., 1996) or absent (Weyrich et al., 1994). These conflicting results may be explained either by species-specific differences or by the different cardiac preparations used, i.e., isolated unloaded myocytes, papillary muscle or intact heart (*in vivo* or *in vitro*), in association with various levels of sympathetic or parasympathetic tone (Balligand, 2000), myocardial cGMP content (Mohan et al., 1996) and interactions between several endothelium-derived mediators such as prostaglandins (Mohan et al., 1995). The independent, and in some cases opposite, effects of cardiac NOS1 and NOS3 (Barouch et al., 2002) make the picture even more complex. In our work, the NO dependent negative inotropic tone was similar in both eel and salmon hearts (Gattuso et al., 2002; Imbrogno et al., 2001) and comparable with the negative inotropy detected in the amphibian (*Rana esculenta;* Gattuso et al., 1999) heart, studied under identical experimental conditions. This is an important

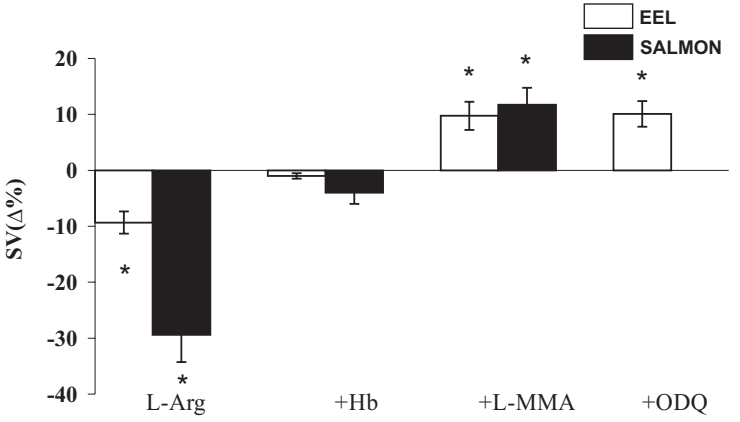

Fig. 15.1 Effects of: L-arginine (L-Arg; 10^{-6}M), alone and in presence of haemoglobin (Hb; 10^{-6} M), N^G-monomethyl-L-arginine (L-NMMA; 10^{-5}M) and 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ; 10^{-5}M) on stroke volume (SV) in isolated and perfused eel and salmon hearts. Percentage changes are shown as means ± S.E.M. of four to six experiments for each drug. The statistical significance of differences was assessed using the paired Student's *t*-test (*=P<0,05).

point and could help to explain some of the conflicting results reported in mammals. In fact, in both fish and amphibian heart preparations, loading conditions and heart rate can be controlled, while the vascular endothelium is either absent (frog) or excluded (eel and salmon) by means of the luminal type of perfusion used.

We have also shown that a NO-cGMP system influences cardiac performance in the cold-adapted Antarctic teleost, the icefish *Chionodraco hamatus*, which is characterized by evolutionary loss of haemoglobin and multiple cardio-circulatory and subcellular compensations for efficient oxygen delivery (Pellegrino et al., 2004). In both eel and icefish hearts, we have detected—by electrochemical assay—the release of NO (in terms of nitrite) in the cardiac effluent and the presence of NOS by morphological methods (i.e., NADPH-diaphorase activity and immunolocalization) (Pellegrino et al., 2004). The presence of a functional NOS system in the hearts of these two teleosts, which differ remarkably in their evolutionary history and ecophysiology, stresses the importance of cardiac NO in fish.

NO AND FRANK-STARLING RESPONSE

According to the Frank-Starling's Law of the heart (heterometric regulation), common to all vertebrate hearts, the end-diastolic volume

and consequent stretch of the myocardial fibres is a major determinant of stroke volume and, hence, cardiac output. When the return of venous blood to the heart (preload) of a fish increases, the lengthened atrial and ventricular myocardial fibres will contract more vigorously and perform more work, hence increasing stroke volume. Fish are particularly sensitive to the Starling response. Indeed, unlike higher vertebrates, in fish the resulting increased cardiac output is achieved mainly through an increased stroke volume rather than heart rate (Farrell and Jones, 1992). Therefore, it is of particular interest to establish whether NO can influence the heterometric regulation in the fish heart. In both the isolated working hearts of *A. anguilla* and *S. salar,* a basal release of endogenous NO affects the Frank-Starling response (i.e., the preload-induced increases in cardiac output at a constant afterload and heart rate) by making the heart more sensitive to filling pressure changes (Fig. 15.2). These data are in agreement with the results obtained in mammalian heart preparations (Prendergast et al., 1997). Influences of NO on either systolic (e.g., mechanisms involving a reduction of intracellular calcium: Shah et al., 1994) or diastolic (e.g., reduction in diastolic stiffness: Paulus et al., 1994) functions have been postulated as underlying mechanisms. The Frank-Starling response contributes to the regulation of cardiac performance in both *in vivo* and *in vitro* hearts by interacting with mechanisms such as heart rate, neurohumoral modulation and coronary flow (see, for mammals, Lakatta, 1992 and for fish, Farrell and Jones, 1992; Imbrogno et al., 2001; Gattuso et al., 2002). Since exercise adjustments in fish generally rely on the Frank-Starling response, our finding that NO significantly influences the intrinsic heterometric regulation in the fish heart may underlie a major evolutionary and adaptative role of cardiac NOS system in these poikilotherm vertebrates.

General consensus exists that inflammation and/or cytokine activation elicit induction of the 'calcium independent' isoform iNOS whose hallmark is the high-output NO production, which in turn may cause tissue and organ (heart failure: Finkel et al., 1992) damage with various mechanisms (Vallance et al., 2000). In trout (*Oncorhynchus mykiss*) challenged with Gram-positive pathogen *Renibacterium salmoninarum,* iNOS transcript expression has been shown in the gills (Campos-Perez et al., 2000). In *Salmo salar* infected with ISA, i.e., 'Infectious Salmo Anemia', a viral disease targeting the vascular and EE cells, we presented evidence of cardiac dysfunction correlated with the

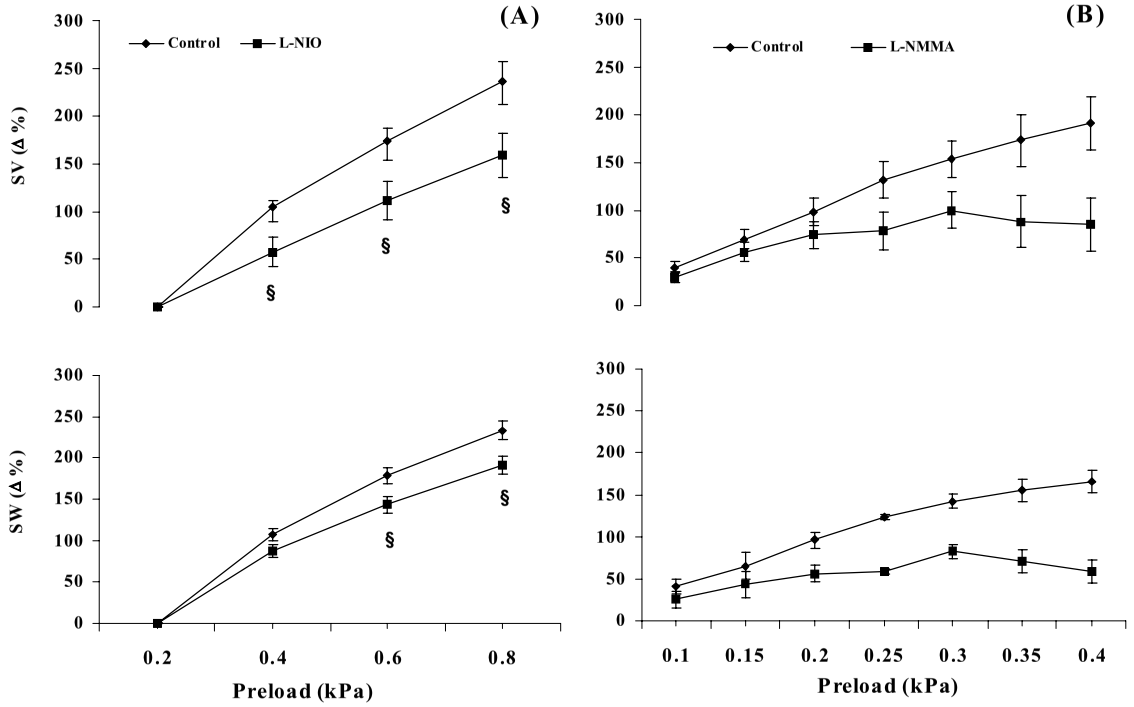

Fig. 15.2 Effects of preload on stroke volume (SV) and stroke work (SW) in control conditions and after treatment with L-N^5(1-iminoethyl)ornitine (L-NIO; 10^{-5} M) in isolated and perfused eel hearts (A) and in control conditions and after treatment with N^G-monomethyl-L-arginine (L-NMMA; 10^{-5}M) in isolated and perfused salmon hearts (B). Percentage changes are shown as means ± S.E.M. of 4 experiments for each group. Comparisons between groups were made using two-way analysis of variance (ANOVA). Significant differences were detected using Duncan's multiple-range test. §Significantly different from the control group ($P<0,05$).

severity of the disease (Gattuso et al., 2002). Although our study provided no direct evidence for iNOS in the pathogenesis of the cardiac dysfunction, the depressed contractile responsiveness to the Frank-Starling response could be completely reverted by an iNOS specific inhibitor (L-NIL) (Gattuso et al., 2002). This result suggests that induction and activation of iNOS may play a part in the pathogenesis of heart failure in infected fish.

NO AND CHEMICAL MODULATION

In the mammalian heart there is compelling evidence that NO, via spatial localization of constitutively expressed Ca^{++} /calmodulin-sensitive NOS isoforms in proximity of cell membrane receptors and ion channels, modulates cardiac function linking extracellular chemical stimuli (neurotransmitters, hormones, autacoids) with appropriate intracellular signalling effectors (Barouch et al., 2002). This NO fine-tuning receptor activity also modulates myocardial contractility. A number of cardioactive agents (histamine, serotonin, glucagon, angiotensin II, parathyroid hormone, etc.) exert their inotropic effects through a regulation of the trans-sarcolemmal L type calcium current (I_{Ca}) which, in turn, triggers cardiomyocyte contraction. There is ample evidence of NO modulation of I_{Ca}. In particular, the NO involvement in the adrenergic (sympathetic) and cholinergic (parasympathetic) neuromodulation exerted by noradrenaline and acetylcholine (ACh), respectively, with the consequent activation of second messengers (cAMP and cGMP) pathways, has been studied extensively (Balligand, 2000). For example, constitutive NOS activity linked to the muscarinic cholinergic signal transduction cascade has been described in the endocardium of several mammalian hearts (see Balligand, 2000, for references). Similarly, interactions between Angiotensin II (ANG II) and eNOS take part in the downstream transduction cascade activated by AT_1 receptor (see for references, Li et al., 2002; Paton et al., 2001). In the absence of other data regarding fish heart, we shall now summarize the involvement of the NO-cGMP pathway in the downstream transduction cascade activated by chemical stimuli such as ACh and ANG II in the *in vitro* working *A. anguilla* heart.

Cholinergic stimulation. Exogenous ACh exerts on the eel heart a biphasic dose-dependent inotropic effect; namely, a positive response at nanomolar concentrations which is mediated by M_1 muscarinic receptors,

and a negative one at micromolar concentrations which is mediated by M_2 muscarinic receptors (Imbrogno et al., 2001). The positive inotropic action of ACh involves a NO-cGMP signal-transduction mechanism. In fact, pretreatment with drugs which block various steps of the NO-cGMP signalling pathway have abolished the positive effects of ACh but did not influence the negative one (Fig. 15.3). A dual mechanism, depending on the NO nanomolar/ micromolar concentration range, has been identified in mammalian and amphibian hearts. For example, in the frog heart, Gattuso and colleagues (1999) have documented that the activation of a NO-cGMP signal-transduction mechanism is necessary to elicit both the positive and negative inotropic responses to ACh. In their study on the isolated ventricular myocytes of the frog (*R. esculenta*), Méry et al., (1993) demonstrated a biphasic *Ica* response to NO donors, which was excitatory or inhibitory, depending on the nanomolar or micromolar ranges of concentration of NO donors, respectively. Both of these stimulatory and inhibitory effects appeared to be mediated by NO and by the corresponding concentrations of cGMP.

Angiotensin II (ANG II) *signaling.* Endoluminal ANG II exerts a direct cardio-suppressive effect on the mechanical performance of the *in vitro* working eel heart via interaction with the AT_1 receptors (Imbrogno et al., 2003). Furthemore, this ANG II-mediated inotropism occurs via an NO-cGMP transduction pathway, since it is enhanced in the presence of the natural NOS substrate L-arginine, while being abolished by the NO scavenger hemoglobin, the NOS inhibitors L-NIO and L-NMMA and the soluble guanylyl cyclase blocker ODQ (Fig. 15.4). Interactions between ANG II and eNOS have been demonstrated, both *in vivo* and *in vitro*, in the mammalian vascular endothelium (see for references, Li et al., 2002), in which the AT_1 subtype receptor has been identified. In contrast, except for a study describing AT_2 receptors in the human EE (Wharton et al., 1998), there are no reports regarding ANG II receptors in the EE. Our data suggest that the interaction between ANG II and EE AT_1 receptors in the eel heart triggers an NO-cGMP signal transduction pathway which, in turn, affects myocardial inotropy. One of the most important targets of the NO-cGMP pathway in cardiac myocytes is cGMP-dependent protein kinase G (PKG). PKG can depress contractility by inhibiting I_{ca} or by depressing the Ca^{++}-sensitivity of myofilaments or phosphorylating the inhibitory subunit of troponin (Hove-Madsen et al., 1996). Since in the eel heart, pretreatment with the PKG inhibitor KT_{5823} attenuates the

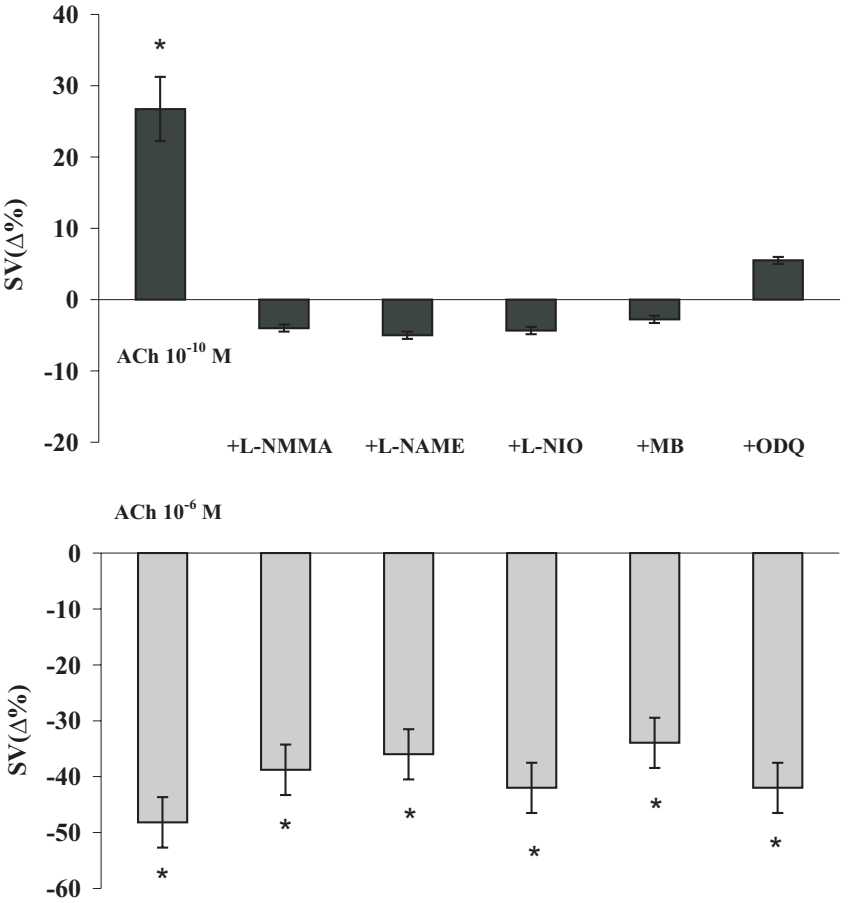

Fig. 15.3 Effects of acetylcholine (ACh; 10^{-10}M and 10^{-6}M) before and after treatment with N^G-monomethyl-L-arginine (L-NMMA; 10^{-5}M), N^G-nitro-L-arginine methyl ester (L-NAME; 10^{-4}M), L-N^5(1-iminoethyl)ornitine (L-NIO; 10^{-5}M), methylene blue (MB; 10^{-6}M) and 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ; 10^{-5}M) on stroke volume (SV) in isolated and perfused eel hearts. Percentage changes are shown as means ± S.E.M. of five experiments for each drug. The statistical significance of differences was assessed using the paired Student's *t*-test (*=P<0,05).

ANG II-mediated negative inotropism (Fig. 15.4), there is evidence of the involvement of PKG in this response.

THE AUTOCRINE-PARACRINE ROLE OF EE

The EE cells, formed shortly after gastrulation from the cardiogenic mesoderm between the endodermal and mesodermal layers, constitute the

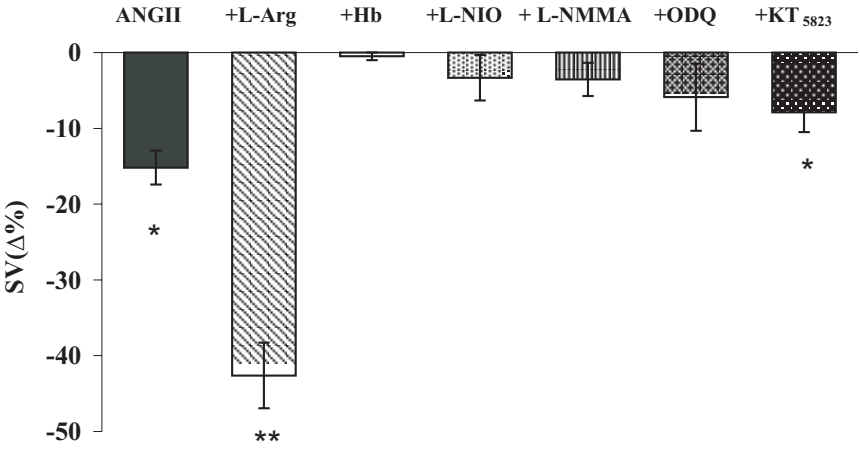

Fig. 15.4 Effects of angiotensin II (ANG II; 10^{-8}M) before and after treatment with L-arginine (L-Arg; 10^{-6}M), haemoglobin (Hb; 10^{-6}M), L-N^5(1-iminoethyl)ornitine (L-NIO; 10^{-5}M), N^G-monomethyl-L-arginine (L-NMMA; 10^{-5}M), 1H-(1,2,4)oxadiazolo-(4,3-a) quinoxalin-1-one (ODQ; 10^{-5}M) and KT_{5823} (10^{-7}M) on stroke volume (SV) in isolated and perfused eel hearts. Percentage changes are shown as means ± S.E.M. of four to five experiments for each drug. The statistical significance of differences was assessed using the paired Student's *t*-test (*=P< 0,05).

single-cell-thick lining of the cardiac chambers. In the vertebrate embryo, the double-walled primary heart tube is made up of only two cell types, i.e., the EE cells of the inner layer and the cardiomyocytes of the outer layer, which are separated by the interposed cardiac jelly, an extracellular amorph matrix. During the double-walled stage of heart development, the EE and the cardiomyocytes together constitute the primitive spongy heart tube (Moorman and Christoffels, 2003). Compelling evidence indicates that important interactions between these two cell types play a major morphogenetic role at different steps in cardiac morphogenesis. For example, EE signalling appears a prerequisite for the process of trabeculation, when beating cardiomyocytes migrate towards the endocardium, producing the myocardial trabeculae network (Brutsaert, 2003; Moorman and Christoffels, 2003). In zebrafish embryos, in which intracardiac flow forces were quantitatively analyzed *in vivo,* the shear forces appear as a relevant factor in cardiac morphogenesis (Hove et al., 2003). It may be argued—from this and other studies—that EE cells may be the components that both sense and transduce these biomechanical stimuli caused by pulsatile blood flow. It has been recently shown that in the adult heart also, the EE plays an obligatory role in controlling

myocardial performance in various mammalian species in an analogous manner to the autocrine-paracrine autoregulation of smooth muscle by vascular endothelium (Brutsaert, 2003). Strategically located between the luminal blood and subjacent cardiac muscle, the EE synthesizes and releases a variety of autocrine-paracrine substances like NO, prostacyclin, ANG II, endothelin, which directly influence cardiac function. In particular, the EE appears to act as a sensor transducing intracavitary physical and chemical stimuli into signaling pathways able to regulate the subjacent working myocardium (Brutsaert, 2003, and references therein).

Most teleost hearts have a fully trabeculated ventricle (i.e., the *spongiosa*), which is supplied only by the luminal intertrabecular (i.e., *lacunary*) system (avascular heart). This complex lacunary surface is completely lined by the EE. Consequently, there is a remarkably high ratio of EE cavitary surface area to ventricular volume (Tota and Gattuso, 1996). In other fish species, the spongiosa is covered by an outer compact layer (i.e., the *compacta*), which is supplied by arterial vessels (coronaries) (Tota and Gattuso, 1996, and references therein). Therefore, in the fully trabeculated fish heart ventricle, the EE surface is the only barrier between the cardiac lumen and the subjacent myocardium and, at the same time, is much larger than in the compact ventricular myoarchitecture of the higher warmblooded vertebrates. We can thus expect that the role of the EE, acting as sensor of the intracavitary stimuli and as autocrine-paracrine modulator of myocardial performance, can be relatively more important in fish than in the homeotherms. NOS system in the EE could take a relevant part in this autocrine-paracrine mechanism. Although more studies are needed to validate this hypothesis, the synopsis illustrated in Fig. 15.5 suggests the presence of this intracavitary EE-NO-dependent regulation of mechanical performance in the fish heart. It is evident that in the *in vitro* working eel heart, treatment with Triton X-100, a detergent that at the concentration used, damages the EE functionally but not structurally, produces a positive inotropic effect probably due to an interruption of the signal transduction pathway which normally activates eNOS in the EE (Fig. 15.5B). Notably, similar finding has been detected in the fully trabeculated frog heart by Sys et al., (1997) who have discussed the possible mechanism whereby Triton X-100 depresses basal release of NO from the EE. An example of the EE role in sensing and transducing chemical stimuli is illustrated by the positive inotropic response induced by nanomolar concentration of ACh. This response requires the functional integrity of the EE, since it is abolished

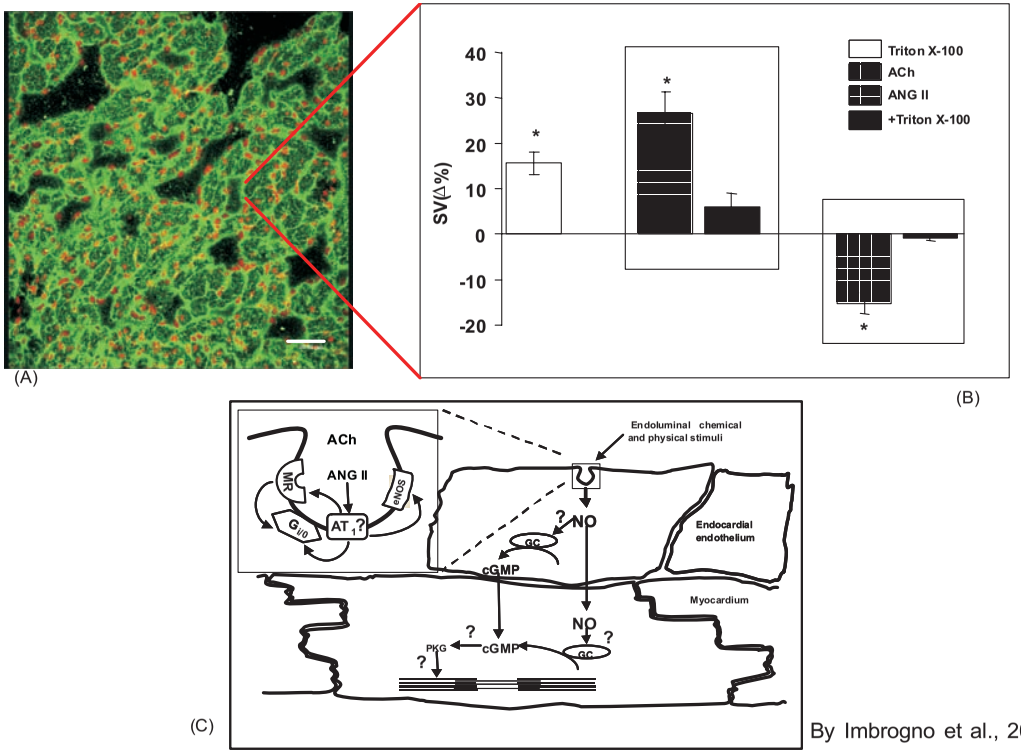

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Fig. 15.5 (A) Immunolocalization of eNOS in the eel heart (own unpublished results); bar: 80 μm. (B) Effects of: Triton X-100 (0.05%) at basal conditions (*left*), acetylcholine (ACh; 10^{-10}M) before and after treatment with Triton X-100 (0.05%) (*middle*) and angiotensin II (ANG II; 10^{-8}M) before and after treatment with Triton X-100 (0.05%) (*right*) on stroke volume (SV) in isolated and perfused eel hearts. Percentage changes are shown as means ± S.E.M. of four to five experiments for each drug. The statistical significance of differences was assessed using the paired Student's *t*-test (*=P<0,05). (C) Cross talk between endocardial endothelium (EE) and myocardium in the eel heart (for details see Imbrogno et al., 2003).

by Triton X-100 (Fig. 15.5B). In mammals, M_2 and M_4 muscarinic receptor subtypes are preferentially located on the myocardiocytes and through adenylate cyclase inhibition elicit negative cholinergic chronotropic and inotropic effects (Hove-Madsen et al., 1996). On the other hand, M_1, M_3 and M_5 receptor subtypes, principally located on the endothelial cells and functionally coupled to phospholipase C and also phospholipase A_2 and phospholipase D, mediate positive cholinergic response (Brodde and Michel, 1999). Interestingly, the positive inotropism of ACh, which in the eel heart is mediated by M_1 receptor subtypes (Imbrogno et al., 2001), is abolished when the EE is functionally damaged. This mimicks the situation obtained when the NO-cGMP mechanism is inhibited (Fig. 15.3), thus supporting an EE-NO-cGMP- dependent signal transduction pathway.

In the eel heart, the intracavitary ANG II signal also appears to be mediated by the EE. In fact, EE impairment caused by Triton X-100 abolishes the ANG II-mediated inotropic effect (Fig. 15.5B). The EE, through a release of NO, participates in the ventricular fine-tuning of the molecular signaling cascade downstream from the stimulation of the ANG II cardiac receptors. This intracardiac cross-talk between EE-NO-cGMP and chemical stimuli suggests that the EE functional integrity is a prerequisite for the transduction of blood-borne chemical signals to the myocardium, thus emphasizing an EE-mediated intracavitary autocrine-paracrine role in the control of fish heart function (Fig. 15.5C). Of note, acylated NOS has been detected in endothelial cell caveolae, which are the location for many proteins involved in signal transduction cascades, including tissue factors, platelet-derived growth factor receptors, muscarinic cholinergic receptors, PKCs, G proteins, G protein-linked receptors, calcium channels, and the plasmalemmal Ca^{++}-ATPase (Feron et al., 1998; Balligand, 2000, and references therein). Therefore, it is reasonable to assume that the co-localization of eNOS and other such proteins, including AT_1 and muscarinic cholinergic receptors, in the restricted space of the caveolae may provide a temporally and spatially 'delimited' domain for signal transduction (Fig. 15.5C).

CONCLUSIONS

Despite the widespread distribution of the NOS system practically among all animal groups, NO comparative biochemistry and physiology in fish has been scarcely studied. Even now, the conditions that elicited this

pleiotropic molecule to evolve as the major modulator of cardio-circulatory functions in early vertebrates as fish are not yet evidenced. Although evaluation of component signaling pathway in isolation cannot provide adequate understanding for the interactive role of cardiac NO, this synopsis, using teleost hearts as a paradigm, indicates that there are deep phylogenetic roots for cardiac NO-cGMP signaling in vertebrates. In fact, it appears that in fish, as in mammals, NO is able to modulate cardiac performance in relation to the changing demands of the organism by beat-to beat regulation (Starling's mechanism), short-term response (phasic control through chemical modulation), and tonic control through the autocrine-paracrine role of the EE. Likely, this EE-NO-dependent regulatory mechanism may uncover an 'ancestral' function of cardiac NO and can simultaneously be seen as being more important in the avascular and fully trabeculated teleost heart than in the compact and vascularized heart of the homeoterms. Although the lack of comparative data limits conjectures about the evolution of NOS system in fish, we suggest that some relevant aspects of cardiac NO signaling share a substantial level of similarity among phylogenetically distant teleosts such as anguilla, salmon and Antarctic notothenioids. Hopefully, this article will stimulate new comparative work and new ideas.

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SECTION 6

Myotropic Hormones

CHAPTER

16

Myotropic Neurohormonal Peptides in Fish

J. Michael Conlon

ABSTRACT

Recent advances in comparative genomics suggest that the families of genes encoding fish neurohormonal peptides have arisen from multiple whole genome and/or whole chromosome duplications on which are superimposed individual gene duplication events. The duplicate genes evolve at different rates but selective pressure has generally acted to conserve the functionally important receptor-binding domains of the peptides. In this chapter, these concepts are illustrated by analysis of the structures and the structure-activity relationships of the multiple molecular forms of the myotropic peptides belonging to the tachykinin, bradykinin, neuropeptide Y, endothelin, vasoactive intestinal polypeptide, and galanin families. Functional analysis focuses upon those studies in which the endogenous peptide has been studied in its species of origin.

Key Words: Tachykinin; Bradykinin; Neuropeptide Y; Endothelin; Vasoactive intestinal polypeptide; Galanin.

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INTRODUCTION

The study of neuroendocrinology in fish has led to several major advances in the realm of 'mainstream' regulatory peptide research (Conlon, 2000). A number of bioactive peptides have been identified for the first time in fish that subsequently have been shown to play important regulatory roles in mammalian physiology and in the pathophysiology of human disease. Important examples include urotensin I, urotensin-II, stanniocalcin, melanin-concentrating hormone and glucagon-like peptide-1. On the other hand, with advances in comparative genomics and proteomics, it is becoming clear that the majority of the genes encoding the regulatory peptides synthesized by mammals arose either very early in vertebrate evolution or were inherited from invertebrate ancestors. Consequently, the complexity and multiplicity of the regulatory peptide system in mammals is reflected in fish.

This article is restricted to a review of the structures, structure-activity relationships and myotropic actions of peptides belonging to the tachykinin, bradykinin (BK), and neuropeptide Y (NPY) families together with endothelin (ET) vasoactive intestinal polypeptide (VIP) and galanin. Evolutionary pressure to conserve the amino acid sequences of these peptides has not been uniform. While the primary structures of ET and NPY have strongly conserved during the rise of the vertebrates, those of VIP and galanin have been only moderately well conserved and the BK-related peptides and tachykinins have evolved rapidly. Consequently, it is often quite inappropriate to study the biological activities of mammalian neurohormonal peptides in fish. This chapter will, therefore, focus largely on those studies in which the myotropic activities of a peptide have been studied in its species of origin.

TACHYKININS

Biosynthesis of Tachykinins

In mammals, the synthesis of the tachykinins, substance P (SP), neurokinin A (NKA), neuropeptide K (NPK) and neuropeptide γ (NPγ) is directed by a single-copy gene (known as Ppta or TAC1) encoding preprotachykinin A. The gene comprises seven exons. Nucleotide sequence analyses of cloned cDNAs from various mammals have identified mRNAs directing the synthesis of four biosynthetic precursors of SP (α-, β-, γ- and δ-preprotachykinin A) that arise from the

preprotachykinin A gene by an alternative RNA splicing mechanism (Carter and Krause, 1990). The mRNA encoding ß-preprotachykinin A is derived from transcription of all seven exons of the gene so that ß-preprotachykinin A contains SP, NKA and its 36 amino-acid-residue NH_2-terminally extended form, NPK. The mRNA encoding γ-preprotachykinin A lacks exon 4 so that γ-preprotachykinin A contains the sequence of SP, NKA and its 21 amino-acid-residue NH_2-terminally extended form, NPγ (Kawaguchi et al., 1986). The mRNA encoding α-preprotachykinin A lacks exon 6, which precisely specifies the NKA region (Nawa et al., 1984), and the mRNA encoding δ-preprotachykinin lacks exons 4 and 6 (Harmar et al., 1990) so that both these biosynthetic precursors contain the sequence of SP only.

A second single-copy gene (Pptb; TAC3) encodes preprotachykinin B, which is the biosynthetic precursor of neurokinin B (NKB). This gene also comprises seven exons (Kotani et al., 1986) but preprotachykinin B contains only the single tachykinin sequence of NKB and overall sequence similarity between the Ppta and the Pptb genes is low. More recently, a third gene-encoding preprotachykinin C has been identified (Zhang et al., 2000; Kurtz et al., 2002). This biosynthetic precursor shows no significant structural similarity with the other preprotachykinins except in the region encoding an 11-amino-acid peptide with limited structural similarity to SP that has been termed hemokinin 1 (HK-1).

In mammals, the biological effects of the tachykinins are mediated through interaction with three discrete and fully characterized receptors, termed NK_1, NK_2, and NK_3. The receptors are defined pharmacologically in terms of the binding affinities of their endogenous ligands so that SP may be regarded as the preferred agonist of the NK_1 receptor, NKA is the preferred agonist of the NK_2 receptor and NKB is the preferred agonist of the NK_3 receptor. The N-terminally extended forms of NKA, NPK and NPγ bind with highest affinity to the NK_2 receptor (Lecci et al., 2000).

Substance P-related Peptides in Fish

Orthologs of SP have been purified and characterized structurally from a wide range of fish from different classes: Australian lungfish *Neoceratodus forsteri* (Liu et al., 2002) (Dipnoi); rainbow trout *Oncorhynchus mykiss* (Jensen and Conlon, 1992a) and Atlantic cod *Gadus morhua* (Jensen and Conlon, 1992a) (Teleostei); pallid sturgeon *Scaphirhynchus albus* (Wang et al., 1999a) and North American paddlefish, *Polyodon spathula* (Wang et

al., 1999a) (Acipenseriformes); spotted dogfish *Scyliorhinus canicula* (Waugh et al., 1993) (Elasmobranchii); sea lamprey *Petromyzon marinus* (Waugh et al., 1994) and river lamprey *Lampetra fluviatilis* (Waugh et al., 1995a) (Agnatha) (Fig. 16.1). In addition, the primary structure of SP may be deduced from the nucleotide sequence of a cDNA-encoding preprotachykinin A from the goldfish *Carassius auratus* (Lin and Peter, 1997).

The amino acid sequence of SP has been rather poorly conserved during the evolution of vertebrates. However, pressure has acted to conserve those residues at the COOH-terminal region of the peptide (Phe^7, Gly^9, Leu^{10}, and Met^{11}) that are known to be important in the activation of tachykinin receptors. Among the fish tachykinins, the Pro^4 residue is invariant and it has been shown that this amino acid is important in conferring selectivity towards NK_1 receptors (Cascieri et al., 1992).

The biological actions of some of the naturally occuring fish SP-related agonists listed in Fig. 16.1 have been studied in their species of origin. Injections of trout SP ([Lys^1, Arg^3, His^5]-SP) into the unanesthetized rainbow trout *O. mykiss* produced an increase in both systemic and coelic resistances leading to hypertension, bradycardia and a decrease in cardiac output (Kågström et al., 1996). Intraarterial injections of high doses (10 – 50 nmol/kg) of dogfish SP ([Lys^1, Arg^3, Gly^5]-SP) into the unanesthetized dogfish *S. canicula* (Elasmobranchii) produced a slight pressor response but low doses were without effect on blood pressure or heart rate (Waugh et al., 1993).

Studies *in vitro* with isolated trout intestinal smooth muscle and the vascularly perfused trout stomach have demonstrated that trout SP increases motility in a concentration-dependent manner (Jensen et al., 1993). Similarly, in isolated preparations of lungfish foregut circular smooth muscle, lungfish SP produced a slow but prolonged tonic contraction. The response of midgut circular smooth muscle was more complex with the peptide increasing the frequency but diminishing the amplitude of spontaneous contractions (Liu et al., 2002). Lungfish SP had only very weak effects upon longitudinal smooth muscle from either foregut or midgut.

Neurokinin A-related Peptides in Fish

As shown in Fig. 16.1, naturally occurring orthologs of NKA have been isolated from the brains of the rainbow trout and Atlantic cod (Jensen and

Substance P-related peptides

```
Human                                    RPKPQQFFGLM
Lungfish                                 K-R-D--Y---
Goldfish                                 K-R-H--I---
Cod                                      K-R----I---
Trout                                    K-R-H------
Sturgeon                                 K---H------
Paddlefish                               K---H------
Dogfish                                  K-R-G------
Sea lamprey                             RK-H-KE-V---
River lamprey                           RK-H-KE-V---
```

Neurokinin A-related peptides

```
Human                                     HKTDSFVGLM
Cod                                       --IN------
Goldfish                                  --IN------
Trout                                     --IN------
Lamprey                                   -F*-E-----
```

Neuropeptide γ-related peptides

```
Human                    DAG***HGQI SHKRHKTDSF VGLM
Goldfish                 SPA***NA-- TR----IN-- ----
Trout                    SSA***NP-- T-----IN-- ----
Bowfin                   SGAPQ*TVPL GR----GEM- ----
Sturgeon                 SSA***NR-- TG--Q-IN-- ----
Shark                    AS-PTQAGIV GR--Q-GEM- ----
```

Scyliorhinin-I

```
Bowfin                                    SKSHQFYGLM
Sturgeon                                  --YHQ-----
Skate                                     A-HDK-----
Dogfish                                   A-FDK-----
Shark                                     A-FDK-----
```

Scyliorhinin-II

```
Dogfish                                SPSNSKCPDGPDCFVGLM
Torpedo                                ----------------
```

Fig. 16.1 A comparison of the primary structures of tachykinins from fish with the corresponding peptides from the human. Orthologs of scyliorhinin-I and-II have not been identified in mammals. (–) denotes residue identity and (*) denotes deletion of a residue.

Conlon, 1992a) and from the brain of the river lamprey (Waugh et al., 1995a). The nucleotide sequence of a preprotachykinin cDNA predicts that goldfish NKA has an identical structure to trout/cod NKA (Lin and Peter, 1997). This peptide showed relatively low affinity and specificity for the NK_2 receptor in rat fundus that was ascribed to the substitution $Asp^4 \rightarrow$ Asn (Badgery-Parker et al., 1993). At this time, there is no evidence for the expression in fish of the Pptb gene, encoding neurokinin B or the Pptc gene, encoding hemokinin 1.

Trout NKA ([Ile^3, Asn^4]-NKA), given intrarterially at a dose of 1 nmol/kg, was equally effective as trout SP in increasing both coelic and systemic vascular resistance in the rainbow trout and was approximately equipotent with trout SP in increasing the dorsal aortic vascular resistance in an vitro perfusion system (Kågström et al., 1996). In contrast, trout NKA was 14 times less potent than trout SP in stimulating the motility of isolated trout intestinal smooth muscle and 28 times less potent in stimulating the motility of the vascularly perfused trout stomach (Jensen et al., 1993).

Neuropeptide γ-related Peptides in Fish

As shown in Fig. 16.1, naturally occurring orthologs of NPγ have been isolated from the tissues of a range of fish [goldfish (Conlon et al., 1991d), rainbow trout (Jensen et al., 1993), bowfin (Waugh et al., 1995b), pallid sturgeon (Wang et al., 1999a), and hammerhead shark *Sphyrna lewini* (Waugh et al., 1995a)]. Evolutionary pressure has acted to conserve only the functionally important COOH-terminal region of the peptide, whereas the amino acid sequence of the NH_2-terminal extension is highly variable. Although NPγ acts as a high affinity agonist for the NK_2 receptor in rat fundus (K_d = 2.5 nM), the goldfish ortholog, termed carassin, has only moderate affinity (K_d = 18 nM) (Badgery-Parker et al., 1993). The substitution $Asp^4 \rightarrow$ Asn within the NKA sequence at the C-terminus of the peptide was considered to be responsible for this reduction in affinity.

Scyliorhinins

The decapeptide scyliorhinin-1, isolated from the intestine of the European spotted dogfish (Conlon et al., 1986a), was the first tachykinin to be purified from a fish (Fig. 16.1). Subsequently, structurally related peptides were isolated from an extract of the stomach of the bowfin

(Waugh et al., 1995b), from the intestine of the pallid sturgeon (Wang et al., 1999b), from the brain of the longnose skate *Raja rhina* (Waugh et al., 1994), and from the intestine of the hammerhead shark (Waugh et al., 1995a). These tachykinins resemble SP only at their COOH-terminal region and, as the nucleotide sequences of the genes or cDNAs encoding their precursors are yet to be determined, the evolutionary relationship of scyliorhinin-I to the mammalian tachykinins is unclear (Conlon, 1999a). In radioligand binding studies, scyliorhinin-1 acts a high affinity agonist for both the NK_1 and the NK_2 receptors in rat tissues (Buck and Krstenansky, 1987). The cardiovascular properties of the bowfin SP-related peptide have been studied in the unaneshetized bowfin (Waugh et al., 1995b). Following bolus injections into the *bulbus arteriosus*, a dose-dependent rise in vascular resistance and arterial blood pressure and fall in cardiac output was seen but there was no change in the heart rate.

Scyliorhinin II, first isolated along with scyliorhinin-1 from an extract of the intestine of the dogfish *S. canicula* (Conlon et al., 1986a), contains a disulfide-bridged cyclic region and terminates in the sequence motif: Phe-Val-Gly-Leu-Met.NH_2 that characterizes NKA (Fig. 16.1). A truncated form of scycliorhinin-II, representing the (3-18) fragment, was purified from the intestine of the ray, *Torpedo marmorata* (Conlon and Thim, 1988). Scyliorhinin-II shows selectivity for the NK_3 receptor in rat tissues (Buck and Krstenansky, 1987) and [^{125}I]-Bolton-Hunter scyliorhinin II has been used as a NK_3-selective radioligand (Mussap and Burcher, 1990). Studies using this radioligand identified a binding site in membranes of dogfish brain and stomach that did not resemble any of the mammalian tachykinin receptors (Van Giersbergen et al., 1991). The rank order of potency to inhibit binding to this site was: scyliorhinin II = scyliorhinin I > SP = NKA >> NKB. The ligand binding properties of a second tachykinin binding site in dogfish tissues resembled more closely those of a mammalian NK_1 receptor. A possible role for scyliorhinin II in osmoregulation in an elasmobranch is suggested by the observation that the peptide stimulates the secretory activity of the isolated perfused rectal gland in *S. canicula*, its species of origin (Anderson et al., 1995). It was speculated that scyliorhinin-II is a hormonal factor that is released by the dogfish intestine in response to salt loading associated with feeding and ingestion of seawater and thereby regulates the rate of chloride clearance by the rectal gland.

BRADYKININ-RELATED PEPTIDES

The Kallikrein-kinin System

The kallikrein-kinin system in mammals involves the sequential action of several well-characterized proteolytic enzymes (Bhoola et al., 1992). Activation of Factor XII (Hageman factor) in blood at the site of tissue injury or *in vitro* by contact with a charged surface results in the activation of plasma prekallikrein and subsequent generation of bradykinin (BK) by the cleavage of high molecular mass kininogen. BK is rapidly degraded, primarily in the pulmonary circulation, by the action of carboxypeptidase N (kininase I) and angiotensin-converting enzyme (kininase II). In humans, alternative splicing of the primary transcript of the kininogen gene gives rise to a second mRNA that directs the synthesis of low molecular mass kininogen. This protein is a substrate for glandular or tissue kallikrein, a serine-protease that is localized predominantly in kidney, pancreas and pituitary, generating lysyl-bradykinin ([Lys^0]-BK), also known as kallidin. [Lys^0]-BK is rapidly converted to BK in the circulation by the action of aminopeptidases. BK may be produced from kininogens *in vitro* by incubation of heat-denatured plasma with trypsin and [Lys^0]-BK may be produced by incubation with porcine pancreatic kallikrein.

Bradykinin-related Peptides in Fish

Evidence is accumulating for the existence of a kallikrein-kinin system in the blood of fish (Conlon, 1999b). Using chromogenic substrates, Lipke and Olson (1990) showed that gill and kidney of the rainbow trout contains kallikrein activity and kininogen and kininases were detected in trout plasma. More recently, a serine proteinase with kallikrein-like substrate specificity has been purified from the pyloric caeca of the black sea bass *Centropristis striata* (Richards et al., 1995). As shown in Fig. 16.2, trypsin-treatment of heat-denatured plasma from the African lungfish *Protopterus annectens* (Dipnoi) generated [Tyr^1, Gly^2, Ala^7, Pro^8]-BK (Li et al., 1998a). The presence of glandular kallikrein-like activity (kininogenase) and kininase in the kidney of P. *annectens* has also been demonstrated (Masini et al., 1996). Incubation of heat-denatured trout plasma with porcine pancreatic kallikrein produced [Arg^0, Trp^5, Leu^8]-BK (Conlon et al., 1996) and, in much lower yield, [Lys^0, Trp^5, Leu^8] -BK (Conlon and Olson, 1993). The trout belongs to the teleost order

Human	RPPGFSPFR
Lungfish	YG----AP-
Cod	R----W--L-
Trout	R----W--L-
Eel	R----W--L-
Bowfin	----W----
Gar	----W----
Sturgeon	M---M----

Fig. 16.2 A comparison of the primary structures of bradykinin-related peptides from fish with human bradykinin. (–) denotes residue identity.

Salmoniformes and the expression of two non-allelic kininogen genes is consistent with the belief that species in this order doubled their chromosomal content (tetraploidization) approximately 50 million years ago. A 52 kDa kininogen, containing the amino acid sequence of [Arg^0, Trp^5, Leu^8]-BK, was isolated from the skin of the Atlantic salmon *Salmo salar* (Ylönen et al., 1999). [Arg^0, Trp^5, Leu^8]-BK was also generated in the plasma of the distantly related teleosts, Atlantic cod *Gadus morhua* (order Paracanthopterygii) (Platzack and Conlon, 1997) and Japanese eel *Anguilla japonica* (order Elopomorpha) (Takei et al., 2001). Treatment of plasma from either the trout or cod with glass beads under conditions previously shown to activate Factor XII in the plasma of mammals did not generate BK so that mechanism by which the kallikrein-kinin system is activated under physiological conditions in these fish remains to be established.

Amongst the phylogenetically ancient ray-finned fishes (Neopterygii), incubation of heat-denatured plasma from the bowfin *A. calva* (Amiiformes) and the longnosed gar *Lepisosteus osseus* (Semionotiformes) with trypsin generated [Trp^5]-BK (Conlon et al., 1995). Bowfin and gar plasma did not contain Factor XII-like enzyme that was activated by contact with glass beads. Similar treatment of heat-denatured plasma from a sturgeon (a hybrid of the shovelnosed sturgeon *Scaphirhynchus platorynchus* and the pallid sturgeon *S. albus*) (Acipenseriformes) generated [Met^1, Met^5]-BK (Li et al., 1998b). The generation of BK in the plasma of a 'lower actinopterygian' (sturgeon) indicates that at least some of the components of the kallikrein-kinin system may have evolved

before the appearance of the neopterygians (gars, bowfin and teleosts). Attempts to generate a BK-related peptide in heat-denatured plasma from the bichir *Polypterus senegalensis* (Polypteriformes) by the action of porcine trypsin and kallikrein have been unsuccessful (J.M. Conlon, unpubl. data). The Polypteriformes are generally considered to represent a highly specialized survivor of the primitive Actinopterygii such that the Acipenseriformes are regarded a sister-group to the Neopterygians and the Polypteriformes are a sister-group to this combined group (Grande and Bemis, 1996). This suggest, as one possibility, that the kallikrein-kinin system arose early in the actinopterygian lineage but after the divergence of the Polypteriformes and the Acipenseriformes. Consistent with this view, attempts to generate BK in the plasma of an elasmobranch, the European spotted dogfish *S. canicula* and in an agnathan, the sea lamprey *P. marinus* have also been unsuccessful (J.M. Conlon, unpublished data).

Bradykinin Receptors

The actions of BK in mammals are mediated through activation of two distinct receptor subtypes (Farmer and Burch, 1992). The widely-distributed B_2 receptor is characterized pharmacologically by the rank order of potency BK > [Arg^0]-BK >> des[Arg^9]-BK. The B_1 receptor is induced in inflammation and is characterized by the rank order of potency: des[Arg^9]-BK > [Arg^0]-BK > BK. In humans, both receptors are located only 23 kb apart on chromosome 14, suggesting that they arose from a localized tandem gene duplication event. However, at the protein level, the receptors show only 36% sequence identity indicating that the genes are evolving very rapidly.

Recent work (Duner et al., 2002) has led to the structural characterization of the first BK receptor from a non-tetrapod. Using a PCR strategy with primers based on sequences in transmembrane regions 3 and 7 of the mammalian B_2 receptor, an intronless clone was isolated from bacterial artificial chromosome library from the zebrafish *Danio rerio*. The zebrafish BK receptor protein comprises 360 amino acids with 35% sequence identity to the human B_2 receptor and 30% to the human B_1 receptor. By way of comparison, the mouse receptors (McIntyre et al., 1993) show 84% and 78% to the orthologous human receptors and the chicken BK receptor (Schroeder et al., 1997) shows only 49% and 31%. The receptor was mapped to linkage group 17 in the zebrafish genome and there are at least 10 genes in zebrafish linkage group 17 that are

orthologous to genes in human chromosome 14. Neighbour-joining analysis with the human angiotensin 2 receptor as outgroup generates a phylogenetic tree in which the zebrafish receptor segregates with the B_2 receptor family rather than the B_1 family.

Actions of Bradykinins in Fish

Bolus intra-arterial injections of synthetic lungfish BK into unanesthetized African lungfish produced dose-dependent increases in arterial blood pressure and pulse pressure with an increase in the heart rate at higher concentrations (Balment et al., 2002). In contrast, bolus intra-arterial injections of mammalian BK, in doses upto 1000 pmol/kg, produced no significant cardiovascular effects demonstrating that the ligand-binding properties of the receptor(s) mediating the cardiovascular actions of lungfish BK in the lungfish are appreciably different from mammalian B_1 and B_2 receptors.

Although the primary structure of cod and trout BK is the same, the peptide elicits quite different cardiovascular responses in the two species. Bolus intra arterial injections of cod BK ([Arg^0, Trp^5, Leu^8]-BK) into unanesthetized cod produced an immediate increase in the ventral aortic pressure that was of relatively short duration (< 5 min) and an increase in heart rate but mammalian BK was without effect (Platzack and Conlon, 1997). The pressor response was reduced in fish treated with prasozin, a non-specific α-adrenergic receptor antagonist and enhanced in fish treated with enalapril, an inhibitor of angiotensin-converting enzyme (peptidyl dipeptidase A), indicating an involvement of catecholamines, but not an activation of the renin-angiotensin system, in mediating the vasopressor action of the peptide in the cod. In contrast to its actions *in vivo*, cod BK caused a relaxation of the celiac artery precontracted with epinephrine. The relaxation was abolished by the cyclooxygenase inhibitor indomethacin, suggesting that the effect is mediated through the release of prostaglandins, but there was no evidence for the involvement of leukotrienes or nitric oxide in the response (Shahbazi et al., 2001).

Bolus injections of trout BK ([Arg^0, Trp^5, Leu^8]-BK) into the dorsal aorta of unanesthetized trout produced multiphasic effects on arterial blood pressure (Olson et al., 1997). An initial pressor response of short duration (1-2 min) was followed by a fall in pressure and then by a sustained rise in pressure lasting up to 60 min. During the second phase (depressor) response, plasma levels of prostaglandin E_2, the prostaglandin

I_2 metabolite, 6-ketoprostaglandin $F_{1\alpha}$ and leukotriene C_4 significantly rose. This fall in blood pressure was abolished by pre-treatment with indomethacin. The third-phase (pressor) response was prevented by pretreatment with prasozin and lisinopril (an inhibitor of angiotensin-converting enzyme) suggesting that this phase is a catecholamine- and angiotensin II-mediated response to the preceding hypotension. Intracerebroventricular injections of [Arg^0, Trp^5, Leu^8]-BK (up to 500 pmol) into unanesthetized trout had no effect on arterial blood pressure or heart rate (Conlon et al., 1996).

Bolus injections of bowfin BK ([Trp^5]-BK) into the *bulbus arteriosus* of unanesthetized bowfin resulted in an immediate fall in arterial blood pressure of 5-10 min duration that was followed by a dose-dependent rise in pressure that was sustained for 30-60 min. There was no change in the heart rate following bowfin BK administration (Conlon et al., 1995). In marked contrast to effects in teleosts, bolus injections of synthetic sturgeon BK in doses as low as 1 pmol/kg into the dorsal aorta of unanesthetized sturgeon resulted in an immediate and monophasic fall in arterial blood pressure with a maximum depressor response at 300 pmol/kg (Li et al., 1998b). Thus, the cardiovascular response of the sturgeon to BK more closely resembles the response of mammals rather than the predominantly pressor response seen in teleost fish. [Met^1, Met^5]-BK produced a strong and concentration-dependent relaxation of rings of vascular tissue from the sturgeon ventral aorta that had been pre-contracted with acetylcholine. The responsiveness of the sturgeon vascular tissues to its native BK was approximately 500-fold greater than the responsiveness of rings of trout epibranchial artery to trout BK.

The isolated longitudinal smooth muscle of the trout stomach and proximal small intestine respond to [Arg^0, Trp^5, Leu^8]-BK with a stable and sustained contraction and the involvement of serotoninergic nerves and arachidonic acid metabolites in mediating its myotropic actions was indicated (Jensen and Conlon, 1997). The actions of trout BK on the motility of trout gastrointestinal smooth muscle are mediated through interaction with a receptor that ligand binding properties that are distinct from either the B_1 or the B_2 receptors of mammals. [Des-Arg^9]-trout BK was a partial agonist but [des-Arg^0]-trout BK and mammalian BK produced no, or only very weak, contractions of trout tissues. The mammalian B_1 receptor antagonist [Leu^8, -des-Arg^9]-BK was without effect on the response of the trout stomach to trout BK and the potent B_2

receptor antagonist, Hoe 140 was a partial agonist. Studies with alanine- and D-amino acid-substituted analogs suggested the hypothesis that the receptor binding conformation of trout BK is defined by the central region (residues 3-7) of the peptide (Jensen et al., 2000).

In contrast to the trout, smooth muscle strips from the stomach of the cod did not respond to cod BK ([Arg^0, Trp^5, Leu^8]-BK) but strips of longitudinal muscle from the cod proximal intestine responded with a concentration-dependent increase in tension (Shahbazi et al., 2001). Experiments with N-terminally and C-terminally truncated analogs of cod BK demonstrate that indicate that the ligand-binding properties of the cod BK receptor are considerably different from the receptor present in trout tissues and may resemble those of the mammalian B_2 receptor more closely. For example, [des-Arg^0]-cod BK was equipotent and produced the same maximum response as cod BK for the contraction of the intestine.

Bolus intra-arterial injections or infusions of eel BK caused significant inhibition of drinking in the seawater-adapted eel, *A. japonica* (Takei et al., 2001). Eel BK produced a similar cardiovascular response in the eel as in the trout with an immediate, transient increase followed by a sustained increase in arterial blood pressure and an initial decrease followed by an increase in heart rate. At the infusion rate of more than 100 pmol/kg/min, plasma concentrations of angiotensin II, a potent dipsogenic hormone in eels, increased, suggesting an interaction of the kallikrein-kinin system and the renin-angiotensin system. In mammals, BK is dipsogenic and vasodepressor so that the peptide exerts opposite effects on fluid and cardiovascular regulation in the eel. The possibility of a physiological role for the kallikrein-kinin system in osmoregulation in marine teleosts is thus suggested.

PEPTIDES OF THE NEUROPEPTIDE Y FAMILY

Evolution of the NPY Family

In mammals, the neuropeptide Y family of homologous peptides comprises three members: NPY, peptide tyrosine-tyrosine (PYY) and pancreatic polypeptide (PP) that are believed to have arisen as a result of a series of gene-duplication events (Conlon et al., 1992b; Larhammar, 1996). Chromosome-mapping studies reveal that the gene-encoding PYY may have arisen from a common ancestral gene (termed NYY) in an ancient chromosomal duplication event that also involved the *hox* gene clusters.

A duplication of the PYY gene concomitant with or just before the emergence of tetrapods generated the PPY gene-encoding PP. In humans, the NPY gene is located adjacent to *hox*A cluster on to chromosome 7p15.1 (Baker et al., 1995), while the human PYY and PPY genes are located only 10 kb apart adjacent to the *hox*B cluster on chromosome 17q21.1 (Hort et al., 1995). This observation has been interpreted as evidence that the initial duplication event leading to separate NPY and PYY genes occurred at the time of a chromosomal translocation whereas creation of the PPY gene involved a tandem duplication of the PYY gene.

NPY-related Peptides in Fish

NPY has been isolated from extracts of the brains of the rainbow trout (Jensen and Conlon, 1992b), Atlantic cod (Jensen and Conlon, 1992b) and European spotted dogfish (Conlon et al., 1992a). The primary structure of the peptide may be deduced from the nucleotide sequence of cDNAs or genomic fragments from the goldfish (Blomqvist et al., 1992), sea bass *Dicentrachus labrax* (Cerdá-Reverter et al., 2000), zebrafish (Söderberg et al., 2000), and the ray *T. marmorata* (Blomqvist et al., 1992) A comparison of the amino acid sequences of these peptides shows that the primary structure of NPY has been strongly conserved, particularly in the C-terminal region, during the radiation of the gnathostomes (Fig. 16.3). In the zebrafish, the *npy* gene is located on linkage group LG19 (LG for linkage group) close to *hox* gene cluster Aa and the *pyy* gene is on a different chromosome (LG03) close to the *hox*Ba cluster (Söderberg et al., 2000). This was interpreted as evidence that the genes arose from a common ancestral gene in a chromosomal duplication event that also involved the *hox* gene clusters. No PPY-like gene could be detected in the zebrafish genomic clone containing the *pyy* gene which is consistent with the view that the PYY-PPY tandem gene pair arose early in the tetrapod lineage.

In marked contrast to its vasoconstrictor action in mammals, NPY from the Atlantic cod relaxed cod celiac arteries precontracted with epinephrine by a mechanism that involved both direct action on smooth muscle and release of prostaglandins but no involvement of nitric oxide (Shahbazi et al., 2002). Cod NPY also produced weak contractions in cod intestinal ring preparations. However, in the elasmobranch *S. canicula*, dogfish NPY was equipotent with porcine NPY in producing concentration-dependent contraction of vascular tissue from the afferent branchial artery (Bjenning et al., 1993).

NPY-related peptides

```
Human              YPSKPDNPGE DAPAEDMARY YSALRHYINL ITRQRY
Sea bass           --V--E---- -----EL-K- ---------- ------
Zebrafish          --T------- -----EL-K- ---------- ------
Cod                --I--E---- ----DEL-K- ---------- ------
Goldfish           --T------- G----EL-K- ---------- ------
Trout              --V--E---- ---T-EL-K- ---------- ------
Dogfish            ---------- G-----L-K- ---------- ------
Torpedo            ---------- G-----L-K- ---------- ------
```

PYY-related peptides

```
Human                YPIKPEAPGE DASPEELNRY YASLRHYLNL VTRQRY
Sea bass             --A--AS-RD G-P----AK- -SA----I-- I-----
Zebrafish            --P---N--D --A----AK- -TA----I-- I-----
Trout                --P---N--- --P----AK- -TA----I-- I-----
Salmon               --P---N--- --P----AK- -TA----I-- I-----
Eel                  --P---N--- --S---QAK- -TA----I-- I-----
Bowfin               --P---N--- --P----A-- -SA----I-- I-----
Gar                  --P---N--- --P----AK- -SA----I-- I-----
Sturgeon 1           A-P---H--D --PA-DVAK- -TA----I-- I-----
Sturgeon 2           F-P---H--D --P-A-DVK-- TA----I-- I-----
Sturgeon 3           F-P---H--D --PA-DVVK- -TA----I-- I-----
Bichir               --P---N--- --P----AK- -SA----I-- I-----
Skate                --P---N--- --A----AK- -SA----I-- I-----
Dogfish              --P---N--- --P----AK- -SA----I-- I-----
```

PY-related peptides from Acanthomorpha

```
Tilapia              YPPKPESPGS DASPEDWAKY HAAVRHYVNL ITRQRY
Sea bass             ---------- N--------- ---------- ------
Anglerfish           ------T--- N-------S- Q--------- ------
Sculpin              ---Q-----G N--------- ---------- ------
```

NPY family peptides from Agnatha

```
River lamprey MPY    MPPKPDNPSS DASPEELSKY MLAVRNYINL ITRQRY
River lamprey PYY    F-------GD N---—QMAR- KA---H---- ------
River lamprey NPY    F-N---S-GE --PA-D-AR- LSA--H---- ------
Sea lamprey MPY      ---------P ---------—- ---------- ------
Aus.lamprey MPY      ---------Q --------—-- -S-------- ------
```

Fig. 16.3 A comparison of the primary structures of peptides belonging to the neuropeptide Y family from fish with the corresponding peptides from the human. PY-related peptides have been identified only in Acanthomorpha.(Teleostei). (–) denotes residue identity. The relationships of the lamprey peptides to human NPY family members are speculative.

PYY-related Peptides in Fish

PYY-related peptides have been isolated from extracts of both brain and stomach from the rainbow trout (Jensen and Conlon, 1992b), from the pancreas of the coho salmon *O. kisutch* (Kimmel et al., 1986) and American eel *A. rostrata* (Conlon et al., 1991b), and from gastroenteropancreactic tissues from a range of phylogentically ancient fish: bowfin, *A. calva* (Amiiformes) (Conlon et al., 1991b), alligator gar *Lepisosteus spatula* (Semiontiformes) (Pollock et. al., 1987), pallid sturgeon *S. albus* (Acipenseriformes) (Kim et al., 2000), bichir *P. senegalensis* (Polypteriformes) (Wang et al., 1999c), and the European spotted dogfish *S. canicula* (Conlon et al., 1991a) and longnose skate *R. rhina* (Conlon et al., 1991b) (Elasmobranchii). The structure of the cDNA-encoding preproPYY is known for sea bass *D. labrax* (Cerdá-Reverter et al., 2000) and the gene encoding PYY for zebrafish *D. rerio* (Söderberg et al., 2000).

On the basis of the data available (Fig. 16.3), it appears that the primary structure of PYY has been much more strongly conserved in fishes than in tetrapods (Conlon, 2002). The hypothesis has been proposed that the more rapid rate of evolution of tetrapod PYY is a consequence of the gene duplication that generated the PPY gene that has thereby relieved the PYY gene of some conservative selective pressure. Cladistic analysis of the amino acid sequences of PYY from gnathostomes has led Larhammar (1996) to propose that the common structure of PYY in the European spotted dogfish (Elasmobranchii), alligator gar, (Semiontiformes) and bichir (Polypteriformes) represents an 'ancestral' sequence from which the other peptides have evolved. The primary structures of PYY from the longnose skate (Elasmobranchii), bowfin (Amiiformes) and rainbow trout (Teleostei) differ from the proposed ancestral sequence by only one amino acid residue. Exceptions to this high degree of conservation among non-tetrapods are the three PYY-related peptides isolated from the pallid sturgeon *S. albus*, which differ from the ancestral sequence by 7 or 8 residues (Kim et al., 2000). This suggests that the genes encoding these peptides have undergone an accelerated rate of molecular evolution, possibly as a result of a tetraploidization event.

Bolus intrarterial injections of the dogfish PYY-related peptide into the unanesthetized dogfish, *S. canicula* produced a dose-dependent increase in arterial blood pressure with a maximum response (67% over mean basal values) elicited by 2 nmol/kg peptide (Conlon et al., 1991a). Studies *in vitro* demonstrated that dogfish PYY produces dose-dependent

contractions of segments of vascular tissue from the dogfish afferent branchial artery (Bjenning et al., 1993). The action of the peptide was not blocked by tetrodotoxin or removal of the endothelium.

PY-related Peptides in Acanthomorpha

Supporting the view that the putative gene duplication that led to the formation of separate PYY and PP genes took place after or concomitant with the emergence of the amphibia (Conlon et al., 1992b; Larhammar, 1996), PP has never been identified in the pancreas of a non-tetrapod species. Peptides with very close structural similarity to the proposed ancestral PYY sequence were identified in the pancreata of the teleost fish, the coho salmon (Kimmel et al., 1986) and the American eel (Conlon et al., 1991b). In contrast, peptides that are structurally similar to each other but appreciably different from the ancestral PYY sequence have been isolated from extracts of the pancreatic islets of the acanthomorph fish, the anglerfish *Lophius americanus* (Andrews et al., 1985), daddy sculpin *Cottus scorpius* (Conlon et al., 1986b) and the tilapia *Oreochromis niloticus* (Nguyen et al., 1995) (Fig. 16.3). The Acanthomorpha constitute the modern, highly derived teleosts that resulted from a dramatic radiation of species in the early Cenozoic (Carroll, 1984). Until recently, the phylogenetic relationship between these peptides (sometimes classified together in the PY family) and other members of the NPY family was unclear. However, nucleotide sequence analysis of cloned cDNAs from a fourth acanthomorph, the sea bass, has shown that this species expresses three distinct genes encoding NPY, PYY, and PY (Cerdá-Reverta, 2000). It is concluded, therefore, that PY gene is not an ortholog of the mammalian PYY gene but is probably the product of an independent duplication of the PYY gene that has occurred relatively late in evolution within the acanthomorph lineage, i.e., the two genes are paralogous.

NPY Family Peptides in Agnatha

The lampreys (Petromyzontiformes), along with the hagfishes, are the only surviving groups from the agnathan phase of early vertebrate evolution. Three members of the NPY family, termed peptide methionine-tyrosine (PMY), have been isolated from the intestines of the river lamprey *L. fluviatilis* (Wang et al., 1999c) and the Australian lamprey *Geotria australis* (Wang et al., 1999c), and from the brain (Conlon et al., 1994) and intestine (Conlon et al., 1991c) of the sea lamprey *P. marinus* (Fig. 16.3).

Current views of agnathan phylogeny favour the hypothesis that the Southern-hemisphere lampreys and the Holarctic lampreys arose from a common ancestral stock but their divergence is of a relatively ancient (pre-Tertiary) origin (Potter and Hilliard, 1987). These peptides, which may represent the agnathan orthologs of PYY, differ from one another by only one or two amino acid residues, indicating that the structure of PMY has been strongly conserved during the evolution of Agnatha.

Molecular cloning studies in the river lamprey *L. fluviatilis* have identified cDNAs encoding an NPY-related peptide together with a second PYY-related peptide that differs from *Lampetra* PMY by 11 amino acid residues (Söderberg et al., 1994). The identification of distinct genes encoding NPY and PYY in the lamprey demonstrates that the putative chromosomal duplication event involving the ancestral NYY gene predates the appearance of the Petromyzontiformes (at least 490 million years b.p.). The genomes of lampreys contain three *hox* clusters (Sharman et al., 1998) suggesting, as one possibility, that a whole or partial chromosome duplication event occurring within the agnathan lineage generated the second PYY gene along with the third *hox* cluster.

ENDOTHELIN

In mammals, endothelin (ET) exists in three isoforms (ET-1, ET-2 and ET-3) that are the products of distinct genes (Rubanyi and Polokoff, 1994). In contrast, only a single molecular form of ET was identified in an extract of the kidney of the steelhead trout, *O. mykiss* (Wang et al., 1999d). Its amino acid sequence shows three substitutions ($Ala^4 \rightarrow Ser$, $Thr^5 \rightarrow Ser$, and $Phe^6 \rightarrow Trp$) compared with human ET-2 demonstrating that the structure of the peptide has been relatively well conserved during evolution and that the pathway of post-translational processing of preproendothelin in the trout is probably similar to that in mammals (Fig. 16.4). ET-3 has been isolated from the tissues of an amphibian [the frog, *Rana ridibunda* (Wang et al., 2000)] and a reptile [the alligator, *Alligator mississipiensis* (Platzack et al., 2002)] so that the failure to identify ET-3 in the trout suggests, as one possibility, that the putative gene duplication events that gave rise to the ET isoforms occurred after or concomitant with the appearance of tetrapods.

Trout ET was shown to be active on the trout cardiovascular system both *in vivo* and on isolated blood vessels. Bolus intraarterial injects of both trout ET and mammalian ET-1 into unanesthetized trout produced

```
Trout ET              CSCATFLDKE CVYFCHLDII W

Human ET-1:           ---SSLM--- ---------- -

Human ET-2:           ---SSW---- ---------- -

Human ET-3:           -T-FTYK--- ---Y------ -
```

Fig. 16.4 A comparison of the primary structure of trout endothelin with the three isoforms of endothelin from human. (–) denotes residue identity.

a monophasic increase in ventral aortic pressure and a triphasic pressor-depressor-pressor response in ventral aortic pressure together with increased central venous pressure, gill resistance and systemic resistance and decreased cardiac output, heart rate, and stroke volume (Hoagland et al., 2000). Increased sensitivity to central venous infusion of ET-1 over dorsal aortic infusion is probably a consequence of the greater exposure of the branchial vasculature to the peptide. Similar cardiovascular effects of ET-1 were seen in the Atlantic cod and it was proposed that the increase in gill vascular resistance was a consequence of pilar cell contraction (Stenslokken et al., 1999). Intracerebro-ventricular (ICV) injection of mammalian ET-1 into unanesthetized rainbow trout elicited a dose-dependent increase in mean arterial blood pressure and a decrease in heart rate that was accompanied by an increase in systemic vascular resistance (LeMevel et al., 1999).

Trout ET produced concentration-dependent constrictions of isolated rings of vascular tissue from trout efferent branchial artery, caeliacomesenteric artery, and anterior cardinal vein. Surprisingly, rat ET-1 was 10- to 20-fold more potent than trout ET in constricting isolated rings of vascular tissue from trout vessels, as well as from rat aorta, but there was no significant difference in the maximum tension produced by either peptide in these tissues. (Wang et al., 1999d). Rat ET-1 is also a potent vasoconstrictor of trout cerebral arteries (Paslawski Rodland and Nilsson, 2002). In the eel, *A. rostrata*, mammalian ET-1 produces constriction of the isolated bulbus arteriosus, a vessel that smoothes cardiac output by expanding during systole and relaxing during diastole (Evans et al., 2003). ET is amongst the most potent constrictors of the trout gill, effectively decreasing lamellar perfusion and the peptide is rapidly metabolised in the microcirculation of this tissue (Olson, 2002).

In the gastrointestinal tract, trout ET produced sustained and concentration-dependent contractions of strips of longitudinal smooth muscle from trout stomach and proximal small intestine and from rat fundus (Wang et al., 2001). Rat ET-1 was equipotent with trout ET for contraction of rat fundus and 2- to 3-fold more potent for contraction of trout gastrointestinal tissues. The actions of ET in mammals are mediated through interaction with two well-characterized receptors (Davenport, 1995). The ET_A receptor is selective for ET-1 and ET-2 whereas the ET_B receptor exhibits similar affinities for all three isopeptides. It is known that the contractile effects of ET-1 on rat fundus are mediated through the ET_B receptor (Gray et al., 1995) and effects on the rat aorta are mediated through the ET_A receptor (Goto et al., 1989). It was suggested, therefore, that trout gastrointestinal tissues express an ET_B -type receptor that differentiates poorly between trout ET and rat ET-1 whereas trout vascular tissues express an ET_A-type receptor that is preferentially activated by rat ET-1. Consistent with this proposal, ET-3 was without effect on cardiovascular parameters when injected either intraarterially or ICV in unanesthetized trout (LeMevel et al., 1999). The ET-induced contractions of the trout gastrointestinal tissues were shown to be, in part, indirect, involving a serotoninergic neuronal pathway in the intestine and a non-cholinergic, non-serotoninergic pathway in the stomach (Wang et al., 2001).

ET from an elasmobranch has not yet been characterized structurally but bolus injections of mammalian ET-1 into the spiny dogfish *Squalus acanthias* produce a fall in arterial blood pressure, reflecting an increase in branchial vascular resistance, that rapidly returned to pre-injection levels (Perry et al., 2001). Effects on the gill vasculature were accompanied by simultaneous decreases in systemic resistance and cardiac output together with persistent hyperventilation. The peptide produced constriction of rings of vascular smooth muscle from the ventral aorta of this species (Evans et al., 1996). The magnitude of the effect was decreased, but not abolished, by removal of endothelium. In contrast to the situation in teleosts, ET-3 was equipotent with ET-1, suggesting the involvement of an ET_B-type receptor. Also in this species, ET-1, but not the ET_B receptor-selective agonist sarafotoxin S6c, produced constriction of rings of smooth muscle from the rectal gland, suggesting that ET may play a role in elasmobranch osmoregulation (Evans and Piermarini, 2001).

VASOACTIVE INTESTINAL POLYPEPTIDE (VIP)

VIP has been purified and characterized structurally from representative of several classes of fish—Atlantic cod *G. morhua* (Thwaites et al., 1989), rainbow trout *O. mykiss* (Wang and Conlon, 1995) and goldfish *C. auratus* (Uesaka et al., 1995) [Teleostei]; bowfin *A. calva* [Amiiformes] (Wang and Conlon, 1995), pallid sturgeon *S. albus* [Acipenseriformes] (Kim et al., 2002), and spotted dogfish *S. canicula* [Elasmobranchii] (Dimaline et al., 1987) (Fig. 5). Two molecular forms of VIP were isolated from the pallid sturgeon differing by one amino acid substitution ($Ala^4 \rightarrow Ser$), which provides some support for the hypothesis that *S. albus*, with approximately 120 chromosomes and belonging to the most basal of the Acipenserinae lineages, is tetraploid (Birstein and DeSalle, 1998). Identity of sturgeon VIP-1 with the peptide isolated from the bowfin and rainbow trout is consistent with the placement of the Acipenseriformes as the sister-group to the Neopterygii comprising the Lepisosteiformes (gars), Amiiformes (bowfin) and Teleostei (teleosts) (Gardiner et al., 1996).

Structure-activity studies measuring the ability of alanine-substituted analogs of pig VIP to bind to the human $VPAC_1$ receptor have shown that substitutions at His^1, Phe^6, Arg^{12}, Arg^{14}, and Leu^{23} resulted in a >100-fold decrease in binding affinity and substitutions at Asp^3, Val^5, Thr^7, Asp^8, Tyr^{10}, Lys^{15}, Lys^{20}, Lys^{21} and Ile^{26} resulted in a >10-fold decrease in binding affinity. Substitutions at other sites have little or no effect on binding affinity and replacement of Ala^4 and Ala^{18} by Gly had no effect on binding (Nicole et al., 2000). In this light, the data in Fig. 16.5 demonstrate that demonstrate that the primary structure of VIP has been moderately well conserved during evolution, with amino acid

```
Human          HSDAVFTDNY TRLRKQMAVK KYLNSILN
Cod            ---------- S-F-----A- -----V-A
Goldfish       ---------- S-Y-----A- -----V-A
Trout          ----I----- S-F------- -----V-T
Bowfin         ----I----- S-F------- -----V-T
Sturgeon 1     ----I----- S-F------- -----V-T
Sturgeon 2     ---SI----- S-F------- -----V-T
Dogfish        ---------- S-I------- --I--L-A
```

Fig. 16.5 A comparison of the primary structures of vasoactive intestinal polypeptide from fish with human VIP (–) denotes residue identity.

substitutions either being conservative or confined to those sites in the molecule that are not involved in receptor interaction. Consistent with this, both cod (Thwaites et al., 1989) and dogfish VIP (Dimaline et al., 1987) are equipotent with porcine VIP in stimulating amylase release from guinea-pig pancreatic acini and goldfish VIP is equipotent with porcine VIP in modulating the short-circuit current across the eel intestine (Uesaka et al., 1995).

The myotropic activities of a fish VIP in its species of origin has not been investigated but porcine VIP produced concentration-dependent *in vitro* relaxation of small arteries from the rainbow trout proximal intestine that was not dependent upon the presence of an intact endothelium (Kågström and Holmgren, 1997). It was proposed that the VIP-induced relaxation was mediated, at least in part, by prostaglandin synthesis.

GALANIN

Galanin, first isolated from an extract of pig small intestine using a chemical assay that detected the presence of a C-terminally α-amidated residue in a peptide (Tatemoto et al., 1983), has subsequently been purified from gastrointestinal tissues of the rainbow trout (Anglade et al., 1994), bowfin (Wang and Conlon, 1994) and pallid sturgeon (Wang et al., 1999b). A truncated form of galanin, identical to the residues (1-20) fragment of bowfin and sturgeon galanin, was also isolated from the stomach of the spotted dogfish (Wang and Conlon, 1994) (Fig. 16.6). Nucleotide sequence analysis of cloned cDNAs from goldfish brain has identified five components encoding preprogalanin that are derived from two different genes by an alternative RNA-splicing mechanism (Unniappan et al., 2003). The primary structure of galanin derived from preprogalanin-1A and -1C is shown in Fig. 6 and may be considered the ortholog of the trout peptide. The predicted galanin from preprogalanin-2A comprises 31 amino acid residues and the predicted galanins from preprogalanin-1B and –2B contain a 24 amino acid insert and so comprise 53 and 55 residues, respectively. The physiological significance of this multiplicity is unknown. The data in Fig. 16.6 demonstrate that selective evolutionary pressure has acted to conserve the N-terminal domain of galanin whereas the C-terminal region is variable. In several mammalian bioassay systems, N-terminal fragments of galanin are equally effective as the intact peptide indicating that this domain represent the primary site of interaction with its receptor(s) (Crawley, 1995).

```
Human        GWTLNSAGYL LGPHAVGNHR SFSDKNGLTS
Goldfish     ---------- -----IDS-- -LG--H-VA
Trout        ---------- ----GIDG-- TL---H--A
Bowfin       ---------- ------D--- -LN--H--A
Sturgeon     ---------- ------D--- SL---H--P
Dogfish      ---------- ------D---
```

Fig. 16.6 A comparison of the primary structures of galanin from fish with human galanin (–) denotes residue identity.

The cardiovascular actions of trout galanin in the unanesthetized trout are dependent upon the route of administration (LeMevel et al., 1998). Intracerebro-ventricular injection (1.0 and 3.0 nmol/kg) of trout galanin produced an increase in mean dorsal aortic blood pressure and systemic vascular resistance without changing heart rate or cardiac output whereas intraarterial injection of the peptide produced dose-dependent decrease in blood pressure and systemic resistance. Intravenous injection of porcine galanin into two species of shark, *Heterodontus portusjacksoni* and *Hemiscyllium ocellatum* produced a rise in caudal arterial blood pressure and *in vitro* the peptide produced contraction of isolated segments of pancreatico-mesenteric artery from these species (Preston et al., 1995).

Trout galanin has little or no effect upon the motility of either circular or longitudinal muscle from the trout stomach and intestine (J. Jensen and J.M. Conlon, unpublished data). However, porcine galanin produced weak, tetrodotoxin-insensitive contractions of intestinal smooth muscle from the Atlantic cod (Karila et al., 1993).

CONCLUSION

Until relatively recently, a cynical commentator might—with some justification—have likened regulatory peptide research to stamp collecting. Structural similarities between peptides in a particular species may have resulted in their being classified together in paralogous families, in much the same way as a philatelist arranges related stamps in his collection, but there was no plausible explanation to account for the different distributions and multiplicities of the family members among the different classes of vertebrates. In particular, the reason for the presence

of numerous 'extra' genes in fish compared with tetrapods was unclear (Wittbrodt et al., 1998). However, dramatic advances in the field of comparative genomics, particularly the elucidation of the genomic maps of the pufferfish *Takifugu rubripes* (Fugu) (Aparicio et al., 2002) and zebrafish *Danio rerio* (Woods et al., 2000), have brought a measure of order to this apparent chaos.

The following scenario, although by no means accepted by all the workers (Hughes et al., 1999; Robinson-Rechavi et al., 2001), provides at least a working hypothesis to account for neurohormonal peptide diversity. The pioneering ideas of Ohno (1970), that were later substantiated by an analysis of the *Hox* gene clusters (Holland et al., 1994), have led to the hypothesis that two rounds (2R) of whole genome duplications occurred in relatively rapid succession immediately prior to, or concomitant with, the emergence of the Agnatha. This concept is gaining increasing acceptance (Gu et al., 2002; Larhammar et al., 2002). These proposed duplications have shaped the genomes of all vertebrates. A further entire genome duplication is believed to have occurred in the ancestral fish lineage, approximately 300-400 Myr ago, that has similarly shaped the genomes of all ray-finned fishes (Taylor et al., 2001; Vandepoele et al., 2004). In selected fish lineages, such as the Salmonids and Catostomids, more recent (25 – 100 Myr ago), independent tetraploidization events may have occurred (Otto and Whitton,, 2000). Superimposed upon these whole genome duplications are tandem or segmental duplications of individual genes or groups of genes that have taken place at different rates in particular vertebrate lineages (Lynch and Conery, 2000). The majority of duplicated genes, whether arising from entire genome or from small-scale duplications, are rapidly deleted or become pseudogenes but some may evolve to encode components with a new functional role (Wagner, 1998). Although the original idea of Susumu Ohno (1970) that gene/genome duplication is the driving force of evolution was based upon somewhat debatable premises, the powerful methods of contemporary molecular biology appear to have validated his insight (Conlon and Harhammar, 2005).

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SECTION 7

Pineal Organ: Structure and Function

CHAPTER

17

The Pineal Organ

Horst-Werner Korf

ABSTRACT

This chapter deals with the pineal complex of fish (lampreys, elasmobranchs and actinopterygians) which serves as a photo-neuroendocrine transducer. In several fish species (e.g., lamprey, pike, zebrafish), the pineal also harbours a circadian (endogenous) oscillator. Signals about the photoperiod are perceived by pineal photoreceptors and transformed either into a neuronal response or a neuroendocrine message. The neuronal response is generated by intrapineal neurons that innervate a variety of diencephalic and mesencephalic brain centres. Most of these also receive retinal input. The neuroendocrine message of the pineal is melatonin, that is rhythmically produced and released during darkness under the control of the arylalkylamine N-acetyltransferase (AANAT). Illumination of the pineal organ acutely suppresses both the formation of melatonin and the neuronal activity. In species endowed with an intrapineal circadian oscillator light stimuli given during darkness, phase-shift the circadian rhythm. Receptors for melatonin are widely distributed in the brain and the peripheral tissues. The effects of melatonin are highly variable and may depend on the species and the environmental conditions. All data

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available to date suggest that the pineal organ of fish acts in cooperation with the retina and extraretinal/extrapineal photoreceptors and forms an important part of a multifaceted time-measuring and time-keeping system.

Key Words: Arylalkylamine N-acetyltransferase; Circadian rhythm; Melatonin; Pineal Photoreceptor; Pineal tract.

INTRODUCTION

The pineal complex of fish (lampreys, elasmobranchs and actinopterygians) is a photo-neuroendocrine transducer; it contains extraocular photoreceptors and serves as luminance detector. Signals about the ambient lighting conditions (photoperiod) are transformed either into a neuronal response or a neuroendocrine message. The neuronal response is generated by intrapineal neurons that receive synaptic input from the pineal photoreceptors and give rise to prominent pinealofugal tracts that innervate a variety of diencephalic and mesencephalic brain centres. The neuroendocrine message of the pineal is melatonin. This indoleamine is rhythmically produced and released during darkness. Melatonin production is controlled by arylalkylamine N-acetyltransferase (AANAT), the rate-limiting enzyme of the melatonin biosynthesis. In several fish species (e.g., lamprey, pike, zebrafish), melatonin biosynthesis is regulated by a circadian (endogenous) oscillator that resides within the pineal complex. Notable exceptions are the salmonids which lack the intrapineal circadian oscillator. In general, illumination of the pineal organ acutely suppresses both the formation of melatonin and the neuronal activity. In those species which are endowed with an intrapineal circadian oscillator, light stimuli given during darkness, phase-shift the circadian rhythm. Receptors for melatonin have been identified in several fish species by receptor autoradiography and molecular cloning and are widely distributed in the brain (e.g., optic tectum, pretectal area, dorsal hypothalamus, hypothalamus, preoptic area and cerebellum) and in peripheral tissues (heart, intestine, gonads and hypophysis). Most of the brain areas endowed with melatonin receptors/binding sites also receive neuronal input from the pineal organ and the retina. Melatonin was reported to affect the timing of reproduction and attenuate ovarian development induced by long-day conditions, but in general, the effects of melatonin are highly variable and may depend on the species and also the environmental conditions. All data available to date suggest that the pineal organ of fish acts in cooperation with the

retina and extraretinal/extrapineal photoreceptors. All these photoreceptive areas appear to establish a multifaceted time-measuring and time-keeping system that allows the fish to anticipate and adapt to environmental changes occurring during the day and the season. In several fish species (particularly in teleosts), the pineal complex was shown to mature earlier than the retina. This indicates that the pineal complex is of highest functional importance during early ontogenetic development when the other constituents of the time-keeping and time-measuring machinery are not yet differentiated.

GROSS ANATOMY AND DEVELOPMENTAL ASPECTS

The highly vascularized pineal complex develops as a dorsal evagination from the roof of the diencephalon between the habenular and posterior commissures (Fig. 17.1). Other neighboring structures are the paraphysis, the velum transversum, the dorsal sac and the subcommissural organ. Pineal development appears to precede the development of other prosencephalic regions, since a member of the emx family homeobox genes which play an essential role in rostral brain development was shown to be first expressed in the zebrafish pineal primordium during ontogenetic development (Kawahara and Dawid, 2002). In most fish, the pineal complex consists of two components: the pineal organ proper (epiphysis) and the parapineal organ. The distal part of the pineal organ proper is located close to the skull, the overlying tissue may form a pineal window characterized by a reduced or lacking pigmentation in the epidermal and meningeal layers and a reduced thickness of the skull. The proximal (stalk) portion of the pineal organ contains a neuronal pathway, the pineal tract that connects the pineal organ with the posterior commissure. The parapineal organ is connected with the left habenular nucleus. As shown in case of zebrafish, the left-sided parapineal organ influences the left-right identity of adjacent brain nuclei, e.g., the left and right habenular nuclei which show consistent differences in size, density of neuropil and gene expression (Gamse et al., 2003). Moreover, the position of the zebrafish pineal organ was shown to depend on asymmetric Nodal signalling in the diencephalon (Liang et al., 2000).

Cyclostomes

The differentiation of the pineal complex shows striking differences between hagfish and lampreys, the major representatives of the

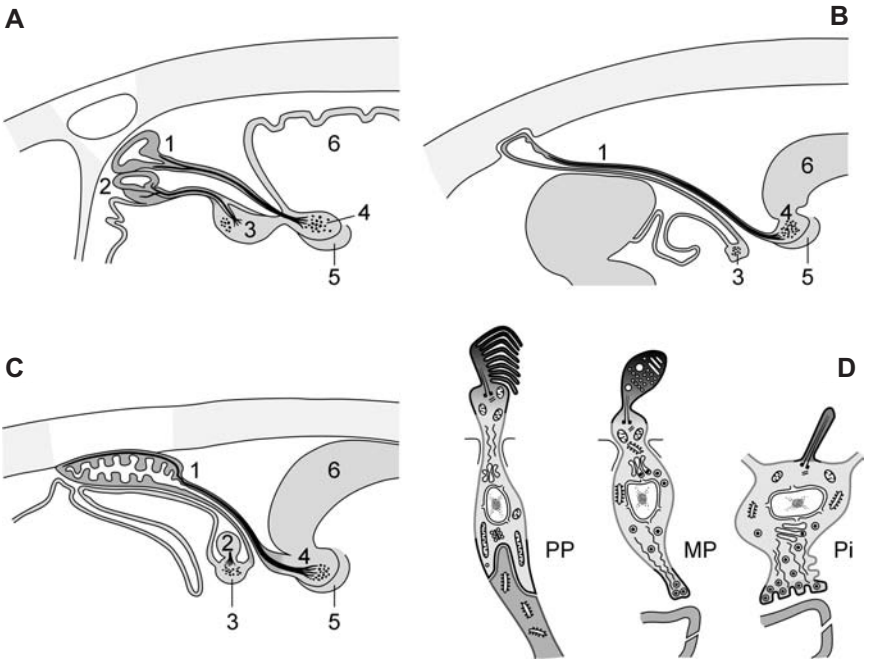

Fig. 17.1 A-C: Topography of the pineal complex of lampreys (A), elasmobranchs (B) and teleosts (C) in the midsagittal plane. 1: Pineal organ proper (epiphysis); 2: parapineal organ (missing in elasmobranchs); 3: habenular commissure; 4: posterior commissure; 5: subcommissural organ; 6: optic tectum. D: Different types of pinealocyte in the fish pineal complex. PP: True pineal photoreceptors, MP: modified pineal photoreceptors; Pi: pinealocytes of the mammalian type.

cyclostome family: whereas a pineal complex is completely lacking in hagfish, it is highly developed in lampreys which possess the pineal and parapineal organs and a specialized pineal window (Fig. 17.1A). The pineal organ comprises the distal end-vesicle, a proximal atrium and a pineal stalk that connects the pineal organ with the brain and harbours the pineal tract, the neuronal connections between the pineal organ and the posterior commissure. The dorsal wall of the pineal end vesicle is much thinner than the ventral wall. The parapineal organ is located ventral to the pineal organ and consists of an end-vesicle and a ganglion that can be considered as an extension of the left habenular nucleus. The parapineal end vesicle contains a prominent lumen; its dorsal wall is much thinner than the ventral wall. As is typical for all fish species, the parapineal organ of lampreys is neuronally connected with the left habenular nucleus.

Elasmobranchs

Elasmobranchs possess a pineal organ only (Fig. 17.1B). In most cases, the organ comprises an elongated stalk and a slightly enlarged distal end vesicle which, however, may be missing in some species (e.g., *Scyliorhinus*). The end vesicle may be attached to the skull. Because of the distal elongation of the pineal organ, the dorsal sac covers the proximal (stalk) part of the pineal only.

Actinopterygians

The pineal complex of most bony fish consists of a pineal and a parapineal organ (Fig. 17.1C). The latter is usually small, located on the left side and connected to the left habenular nucleus. At variance with the lamprey parapineal organ, the parapineal of bony fish is of compact appearance and its lumen is reduced to a capillary space. Chondrosteans have only a pineal organ, whereas most teleosts possess both, the pineal and the parapineal organ. The pineal organ shows striking morphological variation among teleosts, but, in principle, a distal portion (the end vesicle) and a proximal portion, the stalk can be distinguished. The pineal epithelium may be strongly folded, thus narrowing the lumen of the organ. In most cases, the pineal lumen is in open communication with the third ventricle.

CELLULAR COMPONENTS

The pineal parenchyma comprises neuronal elements, i.e., various types of pinealocytes (true pineal photoreceptors, modified pineal photoreceptors, pinealocytes sensu stricto) (Fig. 17.1D) and intrapineal neurons, supportive cells and oligodendrocytes ensheathing the axons of the intrapineal neurons (Fig. 17.2). As shown for zebrafish, generation of neuronal elements in the pineal requires the homeodomain transcription factor Floating head (Flh), which regulates the expression of two basic helix loop helix factor encoding genes *ash1a* (achaete/scute homologue 1a) and *ngn1* (neurogenin 1). The genetic pathways involving *ash1a* and *ngn1* are common to both pineal photoreceptors and intrapineal neurons (Cau and Wilson, 2003). The pineal is highly vascularized, but the pineal parenchyma is separated from the capillaries and the adjacent connective tissue by means of a basal lamina. The capillaries may be either fenestrated (Omura et al., 1985, 1990) or unfenestrated (McNulty, 1976, 1978a, b). Macrophages reside within the pineal lumen and are also found in the pericapillary spaces.

True Pineal Photoreceptors

The principal cell type of the fish pineal organ is the true or typical pineal photoreceptor (Figs. 17.1D, 17.2) which bears an outer segment consisting of a varying number of disks (lamellae), protrudes into the pineal lumen and contains the light-sensitive photopigment. The outer segment disks may form regular cone-shaped stacks resembling those of the retinal cones, but they are often irregular and may comprise a mixture of lamellar and tubular formations. The outer segment is connected to the inner segment via a cilium of the $9 \times 2 + 0$ type. The inner segment contains numerous mitochondria and cytoskeletal elements. The basal process arises from the perikaryon and is directed toward the basal lamina. Its terminals are often enlarged and contain numerous electron lucent vesicles intermingled with synaptic ribbons and scattered dense-core granules. Size, location and numbers of synaptic ribbons vary with the time of the day and the light regimen (McNulty et al., 1988). The terminals of the basal processes contribute to complex neuropil formations and establish synapses with intrapineal second order neurons. A subset of pineal photoreceptors is endowed with very long basal processes that leave the pineal organ and enter the brain (Ekström et al., 1987).

Immunocytochemical and immunochemical investigations have shown that pineal photoreceptors contain proteins of photopigments most of which are closely related to those found in retinal photoreceptors. Thus, immunoreaction for rhodopsin, the protein component of the rod visual pigment rhodopsin has been found in the outer segments of many pineal photoreceptors in lampreys (Tamotsu et al., 1990) and teleosts (Vigh-Teichmann et al., 1982). Molecular investigations have demonstrated the expression of a rod-like opsin (exo-rhodopsin; Mano et al., 1999), vertebrate ancient opsin (Philp et al., 2000) and green-like opsins (Forsell et al., 2001) in the pineal organ of teleosts. The lamprey pineal has been shown to contain a pineal-specific opsin (Yokoyama and Zhang, 1997), originally isolated from the chicken pineal organ and called pinopsin (Okano et al., 1994). Another pineal-specific opsin, called parapinopsin, has been cloned from channel catfish. High parapinopsin expression has been found in cells of the parapineal organ, but the pineal organ contained only a limited number of parapinopsin-expressing photoreceptors which appear concentrated in the proximal portion of the pineal organ (Blackshaw and Snyder, 1997). Taken together, all findings indicate that multiple types of true pineal photoreceptors exist in fish, some have

pineal-specific photopigments, others are closely related to retinal rods and cones. True pineal photoreceptor cells also display immunoreactions for other molecules of the phototransduction cascade, such as alpha-transducin, S-antigen (rod arrestin) and revoverin (Korf et al., 1986). The alpha-transducin immunoreaction is restricted to the pineal outer segments in most cases, the recoverin immunoreaction is located in the perikaryon and the S-antigen immunoreaction labels all compartments of the true pineal photoreceptors including outer and inner segments and basal processes (Korf et al., 1998).

The expression of photopigments and other proteins that are common to the retina and pineal is regulated by cone rod homeobox (Crx)Otx-binding sites. Pineal-specific expression requires in addition to these (Crx)Otx binding sites, a so-called pineal expression-promoting element, PIPE (Asaoka et al., 2002). As shown for another pineal-specific protein, the arylalkylamine *N*-acetyltransferase 2 (see below) pineal-specific gene expression is also controlled by a pineal-restrictive downstream module (PRDM) that has a dual function: enhancement of pineal expression and inhibition of extra-pineal expression (Appelbaum et al., 2004).

The concept that true pineal photoreceptors belong to the neuronal lineage has been supported by the demonstration of neuronal markers in these cells (cf. Korf et al., 1998). As shown for trout pineal photoreceptors, a conspicuous immunoreaction for the neurofilament 200 kDa is present in the axoneme connecting the outer with the inner segment. Similar observations were made in retinal photoreceptors of the rainbow trout, suggesting that neurofilaments form a part of the photoreceptor cytoskeleton (Blank et al., 1997).

The neurotransmitter employed by true pineal photoreceptors has not yet been precisely identified. By means of biochemical techniques high amounts of glutamate and aspartate were detected in the pineal organ of the rainbow trout (Meissl et al., 1978) and goldfish (McNulty et al., 1988c). Immunocytochemistry showed the presence of these two excitatory amino acids in pineal photoreceptors of the goldfish and the Arctic charr (Vigh et al., 1995). It thus appears likely that true pineal photoreceptors employ glutamate or aspartate as neurotransmitters.

Synaptic and neuronal mechanisms are one mode of action of how the fish pineal organ translates the environmental lighting conditions. An additional output mechanism is the production and release of melatonin (see below). In view of these dualistic effector mechanisms, it is relevant

to determine whether true pineal photoreceptors are also able to produce melatonin. This question has been addressed by immunocytochemical investigations using antibodies against serotonin, the precursor of melatonin, and against the hydroxyindole-O-methyltransferase (HIOMT), the last enzyme of the melatonin biosynthesis. (Antibodies against melatonin are of limited value for immunocytochemical investigations since melatonin is a highly diffusible and lipophilic substance that cannot be fixed by conventional chemical fixatives). HIOMT immunoreactivity has been observed in true pineal photoreceptors of several teleost species (Falcon et al., 1994). Pineal photoreceptors were also shown to contain serotonin immunoreaction (Ekström and Meissl, 1997, for references), but considerable species differences do exist. Thus, in the lamprey, true pineal photoreceptors bearing long rod-opsin immunoreactive outer segments lack serotonin immunoreaction, whereas immunoreactive serotonin has been found in two other types of pinealocytes that appear as modified pineal photoreceptors or as pinealocytes sensu stricto (Tamotsu et al., 1990).

Several lines of evidence suggest that pinealocytes may, in addition to melatonin, secrete peptidic or proteinaceous substances whose chemical nature has, however, not yet been elucidated. In this context, findings of Rodriguez et al. (1988) are of interest which have shown that true pineal photoreceptors of the coho salmon (as well as modified pineal photoreceptors of lizards and pinealocytes of rat and bovine display immunoreactions against proteins secreted from the subcommissural organ (ASO and/or AFRU). Although it cannot be ruled out that these immunoreactions are elicited by structural or enzyme proteins, the intracellular distribution of the immunoreactive material, especially its accumulation in the basal pinealocyte processes facing the capillaries or basal lamina may speak in favor of the existence of a secretory protein/peptide produced by a particular population of pinealocytes. This assumption gains further support from the observation that AFRU- and ASO-immunoreactions appear to be exclusively associated with secretory cells of neuroepithelial origin.

Modified Pineal Photoreceptors

Modified pineal photoreceptors are endowed with a rudimentary outer segment which is less regular than the outer segment of true pineal photoreceptors (Fig. 17.1D). Some modified photoreceptors have only a

bulbous cilium lacking membrane disks. The basal process of the cells contain synaptic ribbons intermingled with clear vesicles and dense-core granules. The basal processes of modified photoreceptors terminate adjacent to the basal lamina or are apposed to basal processes of other modified photoreceptors; obviously, these basal processes do not form synaptic contacts with intrapineal neurons. The direct light sensitivity of modified pineal photoreceptors has been proven in birds (Deguchi, 1981), in which they form the main constituent of the pineal parenchyma (cf. Korf et al., 1998; Korf, 1999). Ultrastructural and immunocytochemical investigations revealed the existence of modified pineal photoreceptors also in the fish pineal complex. In pike, they appear concentrated in the caudal part of the pineal end vesicle (Falcon, 1979; Falcon and Collin, 1989). In lampreys, modified photoreceptors are characterized by a strong serotonin immunoreaction and a short outer segment that may display rod-opsin immunoreaction (Tamotsu et al., 1990). The fact that modified pineal photoreceptors are capable of light perception and melatonin production classifies them as photoneuroendocrine cells (Oksche, 1983; Korf et al., 1998; Korf, 1999) that translate information about light and darkness into a neuroendocrine response (i.e., an increased melatonin production during darkness).

Pinealocytes Sensu Stricto

This cell type forms the main cellular component of the mammalian pineal organ (Fig. 17.1D). It is capable of melatonin production, but lacks the direct photosensitivity of true and modified pineal photoreceptors (cf. Korf, 1999). Nevertheless, this cell contains immunoreactions for certain photoreceptor-specific proteins, such as the S-antigen (Korf et al., 1986). Another similarity with true and modified pineal photoreceptors is the presence of synaptic ribbons. The main regulator of pinealocytes in the mammalian pineal organ is norepinephrine (NE). Here, NE is released from intrapineal sympathetic nerve endings, increases the intracellular concentration of calcium ions via stimulation of α-adrenergic receptors and of cyclic AMP via stimulation of β-adrenergic receptors in the pinealocyte membrane and activates melatonin biosynthesis via a cyclic AMP-dependent action on AANAT (Klein, 1985; Klein et al., 1996). Immunocytochemical studies suggest that pinealocytes sensu stricto may also be present in the fish pineal complex. Thus, the lamprey pineal organ contains in its atrium pinealocytes which display a weak S-antigen- and a strong serotonin immunoreaction but lack opsin-immunoreactive outer

segments. It remains, however, to be determined whether these cells in the lamprey resemble mammalian pinealocytes also with regard to the responsiveness to norepinephrine. Investigations of cells isolated from the rainbow trout pineal organ failed to show pinealocytes responding to norepinephrine with an increase in intracellular calcium ion concentration (Falcon et al., 1991; Meissl et al., 1996; Kroeber et al., 1997). Moreover, norepinephrine did not stimulate melatonin production in the trout pineal organ (Meissl et al., 1996). Thus, norepinephrine-sensitive pinealocytes are apparently lacking in the trout. In contrast, such cells may be present in the pike pineal organ, in which AANAT-activity was shown to be controlled by norepinephrine (Falcon et al., 1991).

Intrapineal Neurons and Neuronal Pathways

The fish pineal complex contains a conspicuous population of intrapineal neurons (Fig. 17.2). Their number varies with the species of fish and in some teleosts, e.g., the rainbow trout more than 1500 neurons were counted (Korf, 1974). It has been notoriously difficult to identify such intrapineal neurons by classical neurohistological techniques, e.g., Nissl staining and silver impregnation techniques. The first reproducible results on intrapineal neurons were obtained by the histochemical demonstration of acetylcholinesterase (AChE) in the goldfish (Wake, 1973) and rainbow trout (Korf, 1974). In the trout, multipolar neurons can be distinguished from unipolar ones, but most of the intrapineal neurons could not be classified because they do not display labeled processes. The AChE-positive neurons are 10-15 μm in the end vesicle and 5-8 μm in the pineal stalk. Particularly large neurons (20-25 μm) form a dense accumulation („intrapineal ganglion“) at the tip of the end vesicle. In the pike pineal organ, the intermediate region that harbors the modified pineal photoreceptors lacks AChE-positive neurons. As shown in case of the trout, the parapineal organ comprised numerous, densely packed small neurons; their arrangement resembled that in the habenular nucleus. The teleost parapineal organ may thus be considered as a part of the habenular complex which may comprise scattered S-antigen immunoreactive cells resembling pinealocytes. Intrapineal neurons were also demonstrated by immunocytochemistry using antibodies against neuronal cytoskeleton markers (Ekström and Meissl, 1997) and application of retrogradely transported tracers to the pineal tract (Ekström and Korf, 1985; Ekström, 1987). The tracing studies suggest that virtually all AChE-positive

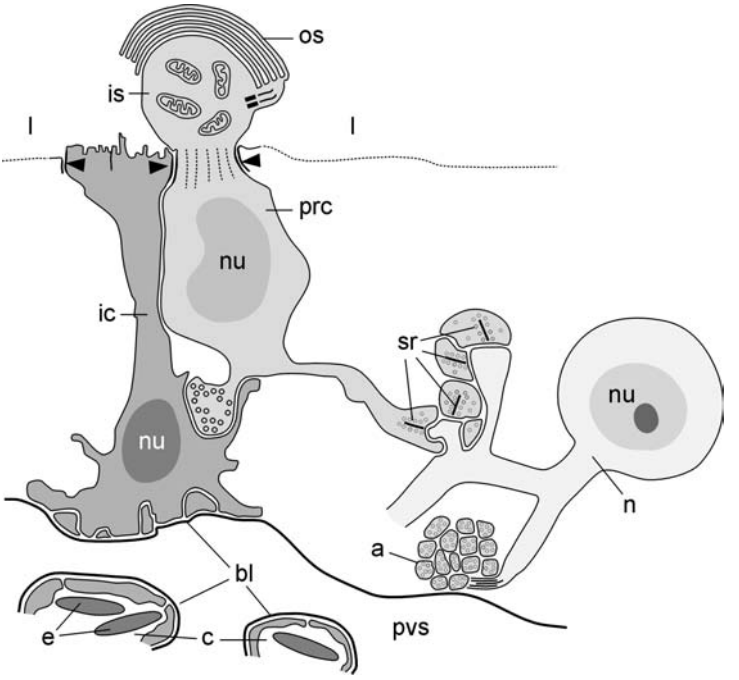

Fig. 17.2 Diagrammatic representation of neuropil formation in the fish pineal organ. a: Axons of intrapineal neurons; bl: basal lamina; c: capillaries; e: erythrocytes; ic: interstitial cell; is: inner segment with mitochondria; l: pineal lumen; n: second-order neuron; nu: nucleus; os: outer segment; prc: perikaryon of the pineal photoceptor cell; pvs: perivascular space; sr: synaptic ribbons in the presynaptic basal pedicles of the pineal photoreceptor; arrowheads: tight junctions between pineal photoreceptors and interstitial cells forming the barrier between the cerebrospinal fluid in the pineal lumen and the pineal parenchyma. Modified after Falcon (1979) and Ekström and Meissl (1997).

neurons send their axons into the pineal tract and are, thus, equivalent to the retinal ganglion cells that form the optic tract. Electron microscopic investigation revealed that neurons forming the pineal tract receive direct synaptic contacts from synaptic ribbon containing terminals of true pineal photoreceptors. These findings argue in favor of a bineuronal chain in the fish pineal organ which consists of the photoreceptor cell as the first neurons and the ganglion cells of the pineal tract as the second neuron. A very limited number of intrapineal neurons were labeled with antibodies against GABA (Ekström et al., 1987) and substance P (Ekström and Korf, 1986), which may represent interneurons. The presence of very few interneurons may also be inferred from the demonstration of conventional synapses on intrapineal neurons. In general, the organization of the

intrapineal neuronal apparatus appears less complex than that of the retina. This is not surprising in view of the fact that the pineal serves as a rather simple luminance detector and is not involved in image generation.

The neuronal connection between the pineal organ and the brain, the pineal tract, may comprise as many as 3000 nerve fibers most of which are unmyelinated (cf. Korf, 1974; Vollrath, 1981; Ekström and Meissl, 1997; Korf et al., 1998). Nerve fibers of the pineal tract are ensheathed by oligodendrocytes which may form a myelin sheath around a limited number of axons. Anterograde tracing techniques (Hafeez and Zerihun, 1974; cf. Ekström and Meissl, 1997; Korf et al., 1998; Yanez and Anadon, 1998; Pombal et al., 1999; Yanez et al., 1999) have shown that the target areas of the pineal projections to the brain follow a basic morphological pattern: they terminate in the reticular formation of the brainstem (central tegmental gray), pretectal area, habenular nuclei, several thalamic and hypothalamic nuclei, and the preoptic region (Fig. 17.3). In elasmobranchs, pineal projections were found in close association with GnRH-immunoreactive neurons in the midbrain (Mandado et al., 2001). The neuronal projections from the parapineal organ may differ from those of the pineal organ. In rainbow trout, the parapineal projects only to the habenular nuclei (Yanez et al., 1996). In two species of lampreys, parapineal projections were found to terminate not only in the habenular nuclei but also in the interpeduncular nucleus (Yanez et al., 1999). Some target areas of pineal projections also receive fiber input from the optic tract. Such a dual innervation may guarantee high precision in transmission of one of the most important environmental zeitgeber, the photoperiod, to the brain. The overlap in retinal and pineal projections also supports the notion that both the retina and the pineal complex cooperate in time-keeping and time-measuring mechanisms.

Several investigations have provided morphological evidence that the pineal tract also comprise pinealopetal nerve fibers originating from various brain areas, such as the habenular nuclei and the mesencephalic central gray (Yanez and Anadon, 1998). In teleosts, the pineal organ is innervated by FMRFamide fibers apparently originating from the nucleus of the terminal nerve (Ekström et al., 1988).

In mammals and birds, the pineal organ receives a dense sympathetic innervation originating from the superior cervical ganglia (cf. Korf et al., 1998, for review and references). This type of innervation employs

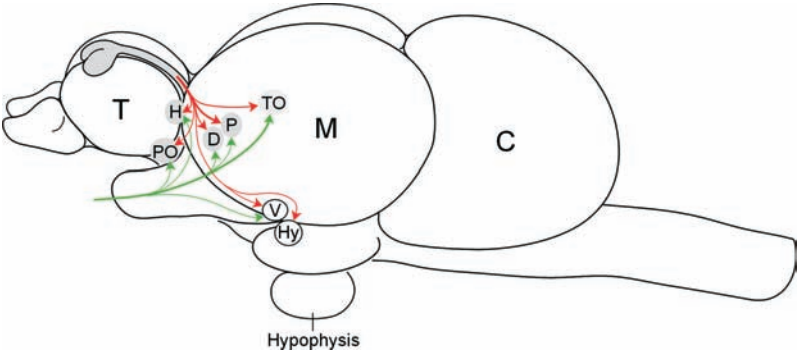

Fig. 17.3 Diagrammatic representation of the pinealofugal and retinofugal neural projections in teleosts. C: cerebellum; D: dorsal thalamic cell groups; Hy: hypothalamus; M: mesencephalon; P: pretectal area; PO: preoptic region; T: telencephalon; V: ventral thalamic cell groups. Modified after Ekström and Meissl (1997).

norepinephrine as primary neurotransmitter and also contains neuropeptide Y. In fish, the sympathetic innervation of the pineal is either absent or much less developed than in birds and mammals. Early studies on the pike with the Falck-Hillarp technique have demonstrated green fluorescent (probably noradrenergic) fibers in the meninges surrounding the pineal organ, but these fibers do not enter the pineal parenchyma (Owman and Rüdeberg, 1970). More recently, Blank et al. (1997) have found NPY-immunoreactive nerve fibers in the capsule and the perivascular space of the pineal organ.

In functional terms, pinealopetal projections, may they be of central or sympathetic origin, appear to be of rather minor importance for regulation of fish pineal since the application of various neurotransmitters or neuropeptides had no or very little effect on the neuroendocrine and/or neuronal activity of the pineal complex.

Interstitial Cells

Interstitial cells (also called supportive cells) form a regular component of the fish pineal organ and are connected to photoreceptors by tight junctions (Fig. 17.2). These establish a barrier between the CSF in the pineal lumen and the more basal parts of the pineal parenchyma that may be exposed to the blood milieu since a blood-brain barrier is lacking (Omura et al., 1985; see below). The basal processes of the interstitial cells line the basal lamina of the pineal parenchyma and form a sheet that

separates neurons and basal processes of pinealocytes from the perivascular space. They are labelled with antibodies against vimentin and glial fibrillary acidic protein (Ekström and Meissl, 1997). There is no indication that interstitial cells are capable of melatonin biosynthesis, although a high synthetic activity may be inferred from the presence of well-developed smooth endoplasmic reticulum and numerous clear and dense core vesicles. In lampreys and other fish species supportive cells display flattened stacks or packed arrays of membranes resembling myeloid bodies of the retinal pigment epithelium. As shown for urodeles, supportive cells incorporate ^{3}H-labelled vitamin A, the most heavily labeled organelles being the myeloid bodies. This suggests an involvement of supportive cells in photopigment metabolism. Furthermore, supportive cells may be engaged in phagocytosis of shedded outer segments. In lamprey, the supranuclear region of supporting cells comprises cristalline structures (Tamotsu et al., 1990) probably containing guanin which supposedly may serve to reflect the light (cf. Vollrath, 1981).

Macrophages

Macrophages are found in the pineal lumen and in the perivascular spaces. In the rainbow trout, these cells were found to take up horseradish peroxidase administered via the blood stream (Omura et al., 1985) and are, thus, able to take up and digest substances penetrating into the pineal parenchyma from the blood stream. Moreover, macrophages in the pineal lumen may be engaged in phagocytosis of shedded photoreceptor outer segments.

BLOOD SUPPLY AND OPEN BLOOD-BRAIN BARRIER

The pineal organ of fish is highly vascularized, but only few studies have dealt with the angioarchitecture (cf. Vollrath, 1981, for review). In the rainbow trout two main arteries (aa. epiphyseales) supply the pineal parenchyma of the rainbow trout. They emerge from the aa. cerebri anteriores and run in the fissure between pros- and mesencephalon. After entering the pineal stalk, they branch off into several arterioles most of which extend into the pineal end vesicle where they give rise to a lobular, bilaterally symmetric capillary network which is drained into two bilaterally arranged veins that are connected either with the vena cerebri anterior or sinus-like veins running at the skull basis. No specialized system of portal vessels was found between the habenular region or the

subcommissural organ (Syed Ali et al., 1987). In most species of fish, the endothelial cells of the pineal capillaries are fenestrated (cf. Vollrath, 1981; Omura et al., 1985) and do not provide a blood-brain barrier. Thus, the basal portions of the pineal parenchyma appear to be exposed to the blood milieu, while the apical (luminal) parts of the pineal parenchyma are exposed to the cerebrospinal fluid. This arrangement is typical of circumventricular organs. Within the fish pineal organ the border between the CSF and the blood milieu is formed by tight junctions between photoreceptor cells and supportive cells.

NEUROPHYSIOLOGICAL ASPECTS

The direct light sensitivity of the fish pineal complex has been proven by electrophysiological methods pioneered by Dodt and coworkers (Dodt, 1963; Meissl and Ekström, 1988; Ekström and Meissl, 1997). Intracellular recordings from pineal photoreceptors have shown that these cells are partially depolarized in darkness and that flashes of bright light hyperpolarize them. Hyperpolarization is usually sustained and monophasic. The relations between the amplitude of the response and the light intensity are very similar in both pineal and retinal photoreceptors, but the intensity range for light flashes is wider for pineal than for retinal photoreceptors. Also, the time course of the responses is different between pineal and retinal photoreceptors. The response of pineal photoreceptors is slower and more prolonged and the time for membrane recovery from peak to dark potentials is also exceptionally long. It, thus, appears that pineal photoreceptors cannot discriminate rapidly changing light stimuli. These characteristics support the notion that the photosensitive pineal organ mediates gradually changing lighting conditions to the circadian organization. The observations that responses to test flashes superimposed on a background illumination are depressed in relation to the background intensity and that pineal photoreceptors undergo major sensitivity changes only during the onset of illumination suggest that pineal photoreceptors serve as luminance detectors. Intracellular recordings and microspectrophotometric measurements have provided evidence that the fish pineal may contain more than one photopigment. This conforms to immunocytochemical results discussed above. Peak sensitivity has been observed to range from 463 to 561 nm. With regard to the neurotransmitters of fish pineal photoreceptors, several studies point toward the excitatory amino acids glutamate and aspartate.

Intrapineal neurons that are postsynaptic to the photoreceptor cells are spontaneously active and their spontaneous discharges are transiently inhibited by brief light flashes. Under steady illumination, the discharge frequency is linearly and inversely related to the intensity of the incident light over a range of almost 6 log units. This so-called achromatic response is most frequently recorded and underlines the role of the pineal organ as a luminance detector. In addition, a so-called chromatic response has been observed in the pineal organ of several fish species. This response is characterized by a long-lasting inhibition of the spike discharge after stimulation with light of short wavelength (blue and UV) and by an excitation after stimulation with light of longer wavelengths (530 or 620 nm). The inhibitory and excitatory responses interact with each other; light of longer wavelengths antagonizes the inhibitory responses to UV and blue light and vice versa. The mechanisms underlying the chromatic response are not yet clarified (see Ekström and Meissl, 1997, for a comprehensive discussion). The chromatic response of the fish pineal organ may provide a switching mechanism during twilight when the spectral composition of the ambient light changes from short to long wavelengths (evening) or from long to short wavelengths (morning).

MELATONIN—THE NEUROENDOCRINE MESSAGE

Melatonin, a lipophilic indoleamine, is produced in the pineal organ and retina of fish and in both organs the photoreceptors are considered as the melatonin-producing cells. Whereas retinal melatonin primarily serves local paracrine functions related to light or dark adaptation, pineal melatonin is released into the general circulation and/or the cerebrospinal fluid and acts as a neurohormone on targets widely distributed in the brain and body. In both the retina and the pineal, melatonin formation follows a clear rhythm, but the maxima of melatonin production occur at different time points: melatonin levels in the retina peak during the day; in the pineal organ, melatonin biosynthesis is activated during darkness and is acutely suppressed by light stimuli. Pineal melatonin can, therefore, be regarded as the neuroendocrine messenger for darkness and a timing hormone.

According to current concepts, the lipophilic melatonin is not stored within the pinealocytes but is released into the blood stream or the cerebrospinal fluid immediately after its formation. Thus, the release of melatonin solely depends on its biosynthesis. As holds true for all

vertebrates, melatonin biosynthesis in the fish pineal organ starts with the uptake of circulating tryptophan into the pinealocytes and involves 5-hydroxylation by tryptophan hydroxylase. 5-hydroxytryptophan is transformed into serotonin (5-hydroxytryptamine) by aromatic L-amino acid decarboxylase. The next step is the formation of N-acetylserotonin catalyzed by arylalkylamine *N*-acetyltransferase (AANAT). Finally, N-acetylserotonin is O-methylated and converted into melatonin by means of the hydroxyindole-*O*-methyltransferase (HIOMT) (Fig. 17.4).

Numerous studies have shown that the rhythm in melatonin production is driven by the rhythmic activation and inactivation of AANAT which can be considered as the rate-limiting enzyme of melatonin biosynthesis and as the molecular interface at which all regulatory stimuli converge. Most interestingly, two forms of AANAT have been identified in teleost fish which are encoded by two different genes and are denominated as AANAT1 and AANAT2. In pike and trout, AANAT1 is found in the retina and AANAT2 in the pineal organ. In zebrafish, AANAT 2 is also dominant in the retina. As shown for the pike, AANAT1 and 2 have distinct differences in their affinity for serotonin and their temperature-activity relations (cf. Falcon et al., 2003b).

An essential requirement for the activation of AANAT is the presence of AANAT protein. The amount of AANAT protein can be regulated at the transcriptional and post-transcriptional level. Transcriptional regulation of AANAT protein is achieved by rhythmic activation of the *Aanat* gene, resulting in rhythmic changes of *Aanat* mRNA levels. This apparently involves an E-box regulatory site in the promoter of the *Aanat* gene (Gothilf et al., 2002). The E-box represents a binding site for the clock gene proteins CLOCK and BMAL1 and mediates rhythmic expression of clock-controlled genes. Rhythmic changes in *Aanat* mRNA levels have been observed in the pike and zebrafish pineal organ, but not in the trout pineal organ in which *Aanat* mRNA levels are continually evelated (Begay et al., 1998). Interestingly, the pineal organs of zebrafish and pike contain an endogenous oscillator and the rhythm in melatonin biosynthesis persists in constant darkness. Similar results were obtained with the ayu (Iigo et al., 2004). In contrast, the trout pineal organ lacks the endogenous oscillator and melatonin biosynthesis is solely controlled by environmental lighting conditions. These findings gave rise to the hypothesis that transcriptional control of *Aanat* occurs only in those species whose pineal organs contain a circadian

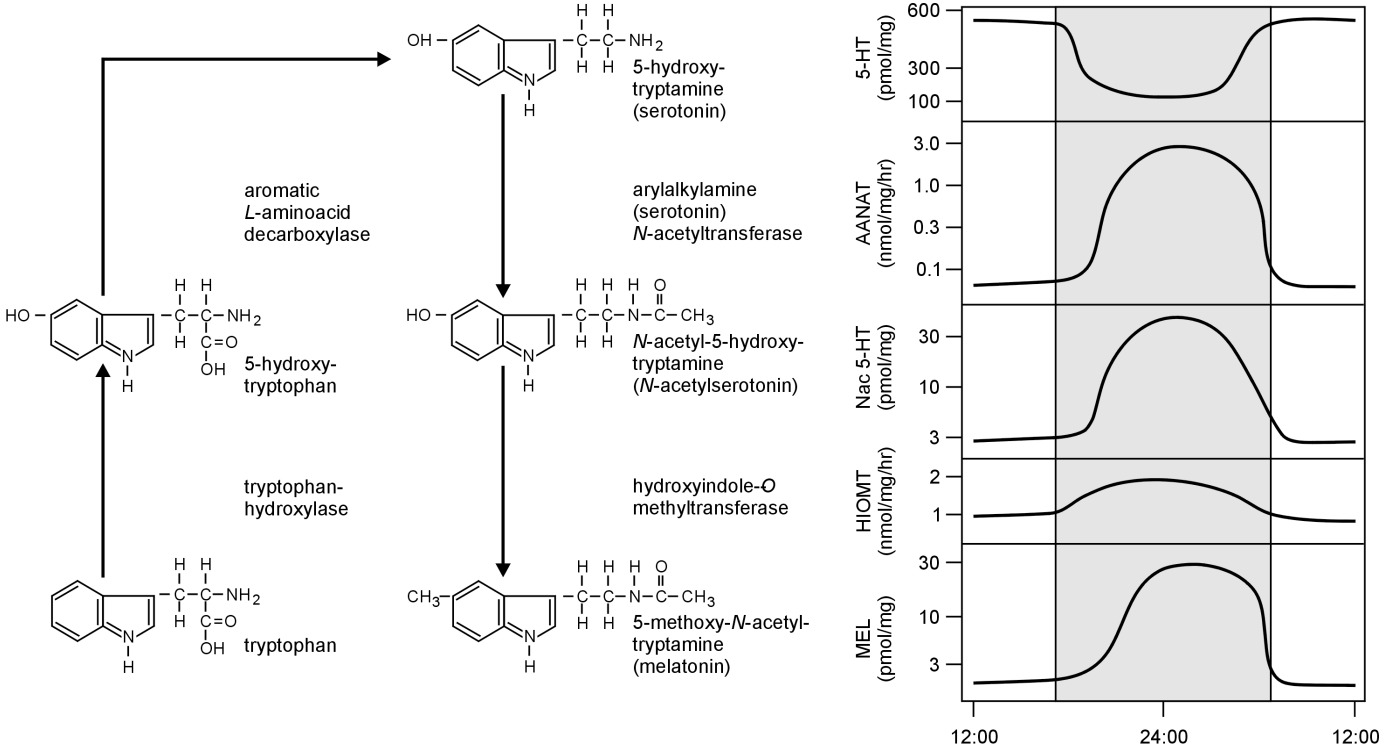

Fig. 17.4 Left: Steps and enzymes of the melatonin biosynthesis. Right: diurnal rhythms in the concentrations of serotonin (5-HT), N-acetylserotonin (Nac 5-HT) and melatonin (MEL) as well as in the activity of the arylalkylamine *N*-acetyltransferase (AANAT) and the hydroxyindole-*O*-methyltransferase (HIOMT). After Klein (1985).

oscillator. At variance with the circadian oscillators in the avian pineal organ (Nathesan et al., 2002) and mammalian suprachiasmatic nucleus (Okamura et al., 2002), very little is known about the molecular machinery of the circadian oscillator in the fish pineal organ (see Cahill, 2002), but the presence of an E-box in the *Aanat-2* gene (see above) suggests that it may composed of transcriptional/translational feedback loops between clock genes similar to those in the avian pineal and mammalian suprachiasmatic nucleus. Otx5, a member of the orthodenticle homeobox family, was shown to regulate circadian gene expression in the zebrafish pineal (Gamse et al., 2002).

Post-transcriptional regulation of AANAT protein levels may depend on the phosphorylation state of the AANAT protein and may involve a controlled degradation of unphosphorylated AANAT protein by proteasomal proteolysis (Falcon et al., 2001). Proteasomal proteolysis controls the amount of AANAT protein in the pike, but its role for the control of AANAT protein amounts in the rainbow trout is not clear. One study reported an increase in AANAT protein amount after inhibition of proteasomal proteolysis (Falcon et al., 2001), while another study failed to show significant changes of AANAT activity after application of inhibitors of proteasomal proteolysis (Kroeber et al., 2000). For mammals, it has been demonstrated that proteasomal degradation of phosphorylated AANAT protein is prevented because the AANAT protein binds to 14-3-3 protein (Ganguly et al., 2002). This binding also increases the affinity of AANAT for low concentrations of serotonin. It may be speculated that interactions between 14-3-3 protein and AANAT protein also play an important role for regulation of pineal AANAT in fish, but to date this hypothesis has not been proven experimentally in any fish species.

Other enzymes of the melatonin biosynthesis, e.g., HIOMT and tryptophan hydroxylase, show at most marginal fluctuations over a 24 h cycle. In some species, HIOMT activity was shown to vary over the year; these changes may contribute to the seasonal modulation of the melatonin biosynthesis. Tryptophan hydroxylase mRNA was shown to cycle in the pike pineal organ which contains a circadian oscillator (see Ekström and Meissl, 1997, for references).

The intracellular signal transduction pathways that regulate melatonin biosynthesis and AANAT activity are only partly understood. Several authors have pointed out that cAMP is the essential second messenger that regulates AANAT protein levels and activity via

activation of protein kinase A (PKA). This conclusion is primarily based on the observations that pharmacologically induced increases in the cAMP concentration (via activation of the adenylate cyclase by forskolin and/or via inhibition of the phosphodiesterase) result in a strong stimulation of melatonin biosynthesis in all fish species examined. A role of cyclic AMP for regulation of the melatonin biosynthesis may also be inferred from the fact that AANAT of pike and trout pineal contains at least to PKA phosphorylation sites that are well conserved in the course of evolution. However, the observation that cyclic AMP levels in the trout pineal organ do not vary between light and darkness whereas melatonin production displays huge differences (Kroeber et al., 2000) argues against a major role of cyclic AMP for the regulation of melatonin production, at least in this species. As shown for the trout, calcium ions appear to play an essential role as second messengers that regulate melatonin biosnythesis in the fish pineal organ (Kroeber et al., 2000, cf. also Begay et al, 1994; Meissl et al., 1996; Krober et al., 1997). The intracellular calcium concentration in fish pineal photoreceptors is controlled by voltage-gated channels of the L-type. Moreover, a cGMP-gated channel may be involved (Decressac et al., 2002). The downstream events of the calcium-dependent signalling pathways that control melatonin synthesis in the fish pineal organ remain to be identified.

There is overwhelming experimental evidence that light and darkness are the dominant regulators of pineal melatonin production either via direct effects on the melatonin biosynthesis or via a phase-shifting effect on the intrapineal circadian oscillator that is present in several fish species. Melatonin biosynthesis is also regulated by temperature whose main influence under natural conditions may be to gate melatonin biosynthesis by control of enzyme kinetics, thereby modulating the synthesis profile which changes over the year (see Ekström and Meissl, 1997, for review and references). In addition, melatonin biosynthesis may be modulated by neurotransmitters and other regulatory factors, such as GABA, catecholamines, acetylcholine, neuropeptides, adenosine and sex steroids. Some of these substances also influence the electric activity of the intrapineal neurons (see Ekström and Meissl, 1997, for review and references). In general, however, non-photic stimuli appear of only marginal importance for regulation of melatonin production in the fish pineal organ.

Melatonin Targets

Specific high-affinity binding sites for iodo-melatonin have been demonstrated in the brain of some species (salmonids, pike, gold fish and gilthead seabream). These binding sites are widely distributed in the brain and occur in centers that process light stimuli and receive also neuronal input from the pineal and retina, e.g., the optic tectum, the pretectum and the dorsal thalamus (Fig. 17.5). Moreover, melatonin binding sites were found in the hypothalamus, in the preoptic area, in gustatory centers that may control feeding behavior and in the cerebellum. As shown for trout and chum salmon, the melatonin receptors in the brain apparently belong to the Mel1a and Mel1b receptor subtype (Mazurais et al., 1999; Shi et al., 2004). Studies with the masu salmon confirmed that the melatonin receptors in the fish brain are coupled to a G-protein (Amano et al., 2003) and revealed that the density of melatonin binding sites is affected by gonadal maturation. In the goldfish brain, the density of melatonin binding sites reached a peak around the light offset and a trough 2 hours before the light onset (Iigo et al., 2003). This rhythm persisted in constant darkness, thus indicating that the density of melatonin-binding sites in goldfish brain is regulated by the circadian clock. Melatonin-binding sites are also reported to occur in peripheral organs, such as the heart (Pang et al., 1994), intestine and gonads (see Ekström and Meissl, 1997).

Physiological Role of Melatonin

Melatonin plays a role in the timing and control of a number of biological rhythms and elicits diverse effects which may differ with the species and the photoperiodic history of the animals (see Ekström and Meissl, 1997, for a comprehensive discussion). Melatonin was reported to affect the timing of reproduction and attenuate ovarian development induced by long-day conditions, but again the effects of melatonin are highly variable and may depend on the species and also on the environmental conditions. In trout, melatonin was found to modulate the secretion of growth hormone and prolactin from the pituitary gland, apparently via a direct action on pituitary cells (Falcon et al., 2003a). Recent investigations with the Atlantic cod confirmed that melatonin as well as norepinephrine are involved in the regulation of pigment aggregation in fish melanophores (Aspengren et al., 2003). Notably, melatonin appears to be involved in night time blanching but not in background adaptation. The diversified effects of melatonin support the concept that the pineal organ of fish acts

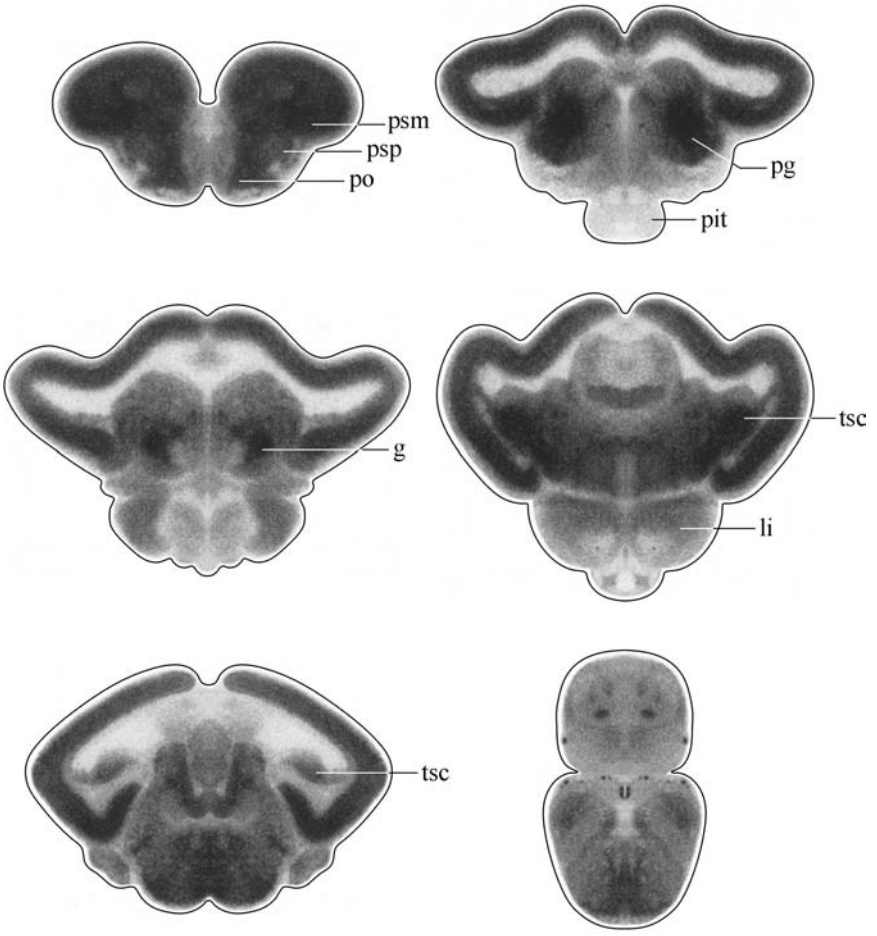

Fig. 17.5 Semidiagrammatic representation of melatonin binding sites in the brain of the Atlantic salmon. g: Nucleus glomerulosus; li: Lobus inferior hypothalami; pg: Nucleus praeglomerulosus; pit: pituitary; po: Nucleus praeopticus; psm: Nucleus praetectalis superficialis magnocellularis; psp: Nucleus praetectalis superficialis parvocellularis; tsc: Torus semicircularis. Modified after Ekström and Vanecek (1992).

in cooperation with the retina and extraretinal/extrapineal photoreceptors. All these photoreceptive areas appear to establish a multifacetted time-measuring and time-keeping system that allows fish to anticipate and adapt to environmental changes occurring during the day and the season. In several fish species (particularly in teleosts) the pineal complex was shown to mature earlier than the retina (Ekström and Meissl, 1997). This indicates that the pineal complex is of highest functional

importance during early ontogenetic development when the other constituents of the time-keeping and time-measuring machinery are not yet differentiated. In line with this concept is the observation that the pineal organ is the first site where a member of the emx family homeobox genes which are essential for rostral brain development is expressed (Kawahara and Dawid, 2002).

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SECTION 8

Stress Response, Reproduction and Endocrine Disruptors

CHAPTER

18

Morphofunctional Aspects of Reproduction from Synchronous to Asynchronous Fishes - An Overview

Maria João Rocha[2] and Eduardo Rocha[1]

ABSTRACT

Viable offspring by sexual organisms results from both sexual and natural selection. Efficiency is reached through a broad spectrum of tactics that enable individuals to attain their reproductive goal, favoured by selection and encoded in each species genome. Photoperiod, rainfall, floods, water temperature, dilution of electrolytes, oxygen content, pH, and lunar cycles, all these factors affect fish reproduction. Fishes interplay their morphophysiology with environmental cycles, resulting in seasonal reproduction. Environmental signs for the approaching favourable breeding time are perceived by exteroceptors, and through a cascade of events ultimately influence the gonads. Therefore, the majority of fishes are seasonal breeders; a few breed

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continuously or, on the contrary, only once during their lifetime. Hypothalamus-pituitary axis involvement is the rule, starting by releasing hypothalamic gonadotropin-releasing hormones (GnRHs). All GnRH forms are potent gonadotropin hormone (GtH) inducers, which are synthesized by two different pituitary gonadotropes. Their release stimulates the production of sexual hormones, such as testosterone (T), and its aromatization to 17ß-estradiol (E_2). Under E_2 stimulus, the liver produces vitellogenin, which is sequestered by the oocytes in process enhanced by GtH-I. In oogenesis, germ cells produce gametes and somatic cells, which later form follicular layers. Oogonia enter meiosis, turning into primary oocytes, which enlarge by way primary growth. Secondary and tertiary growths enlarge the oocyte, with increasing yolk. Maturating inducing steroids (MIS) derived from follicle cells induce meiotic maturation. Follicle atresia occurs, but little is known about it. Four main ovary development patterns exist: synchronous, group-synchronous, multiple-batch group-synchronous and asynchronous. When synchronous, all oocytes grow and ovulate in unison. Such ovaries are found in teleosts that spawn once and then die. The levels of E_2 and T peaked at, or near, the beginning of ovulation. Fishes with group-synchronous ovaries are seasonal breeders, in general, and at least two populations of oocytes can be distinguished at some time. In general, fishes living in temperate regions have a multiple-batch group-synchronous ovary, and undergo multiple ovulations within days or weeks. During the year, levels of all sexual hormones are relatively low. The majority of fishes living in tropical environments are either multiple-batch group-synchronous or asynchronous. In the latter, oocytes of all stages are present without noticing the dominant populations. Even when asynchronous species are sexually ripe, their sex-steroids levels are almost constant. As to the testis, teleosts show two basic microanatomies: lobular and anastomosing tubular (the former being typical for higher teleosts). Atherinomorphs have a special lobular structure (restricted spermatogonial testis type). Spermatogenesis occurs within roundish cysts formed by Sertoli cells. In elasmobranches, spermatocyst development is initiated in a superficial germinal zone. In any case, cysts form by spermatogonial mitosis, which originate spermatocytes that, undergoing the first meiotic division, originate secondary spermatocyes. These then complete the second meiotic division and originate spermatids, finally differentiating into spermatozoa. Duration of spermatogenesis is species specific. The Leydig cells rest in the connective tissue and vary much in number among species, being their primary function to provide steroids needed for gametogenesis and secondary sex characteristics. In elasmobranches, a role for Sertoli cells in steroid production was proposed. Sperm production in some teleosts is a single synchronous event, whereas in others it is cyclic or even truly continuous, being the endocrine control of male reproduction quite similar among species. Testis

maturation coincides mainly with increased levels of 11-ketotestosterone (11-KT). Testicular androgen receptors (ARα and ARβ) seem to play different roles in spermatogenesis. Similar to the female mechanism, an increase in plasmatic GtH-II at the spawning season causes a shift from the steroidogenic production of androgens by the testes towards MIS production. Hermaphroditism occurs naturally in fish, appearing in three forms: protogyny, protandry, and simultaneous hermaphroditism. Histology has revealed that gonads of either protandrous or protogynous hermaphrodites possess some gametes of the opposite sex. Both-ways sex change occurs in several polygynous species. Reversed sex change can also occur in fishes that are protogynous in nature. Few studies reported the role of sex steroids in natural sex differentiation and gender control of hermaphroditic fish, but results suggest different strategies for controlling sex change. Unisexual fishes are rare, but there are examples where females produce only female offspring. Apart from the hypothalamic-pituitary-gonadal axis, the liver is crucial in breeding-related morphofunctional changes. In females, the liver produces both yolk precursors and zona radiata proteins. Under sex-steroid control, hepatocytes have their histophysiology of reproduction (in males too). How the hepatic metabolism is differentially controlled from synchronous to asynchronous species, remains largely unexplored.

Key Words: Fish; Breeding; Synchronous; Asynchronous; Hypothalamic-pituitary-gonadal axis; Ovary; Testis.

INTRODUCTION

The production of viable offspring by sexual organisms is the outcome of both sexual and natural selection (Conover and Heins, 1987; Bell, 1996). Given the very different parts of the globe in which fish propagation occurs, it is expected that their breeding patterns vary (Redding and Patiño, 1993). Actually, each species did develop the particular approach of breeding that promoted its survival, with the maximum reproductive production being reached through the so-called reproductive strategy, i.e., a complex adaptation consisting of a broad spectrum of tactics that enable individuals to attain their reproductive goal (Stearns, 1992), favoured by selection and encoded in the genome of each species (Bell, 1997).

Considering that environmental conditions are set either on diurnal, lunar, annual or astronomical cycles, as well as on regional geographic characteristics, it is commonly accepted that these factors control the reproductive physiology of fishes (Conover and Heins, 1987; Redding and Patiño, 1993). In fact, several studies demonstrate that populations of the

same species, living at different latitudes, compensate for differences in thermal environment, and seasonality, by adjusting the response of sex ratio to temperature, and by changing the level of environmental influence as opposed to genetic control (Conover and Heins, 1987). Thus, it is consensual that amongst vertebrates, fishes contain the greatest variability of sex-determining mechanisms, including environmentally controlled sex determination (Conover and Heins, 1987), hermaphroditism (Sakakura and Noakes, 2000), unisexual and bisexuality (Price, 1984).

In this chapter, we make a to-the-point overview about the *modus operandi* of fish hypothalamus and pituitary as well as some considerations about the role played by different environmental stimuli. The way these facts influence the gonads and their different patterns of development is systematically reviewed, and recent information about different strategies of reproduction is included. Finally, a brief note reports the vital contribution of the liver for fish reproduction. We have related the structure and function, with emphasis in selected aspects. The reasonably extensive reference list was carefully selected to cover a broad time frame and to help readers reach what we consider proper works for further insights.

THE HYPOTHALAMUS-PITUITARY AXIS IN FISH REPRODUCTION

Common features exist in the general organization and detailed morphology of the hypothalamus and pituitary among fishes. This occurs, in spite of the existing wide tactics of breeding.

Therefore, the fish brain controls reproduction via the release of gonadotropin-releasing hormones (GnRHs) (Yu et al., 1997). The GnRHs are decapeptides produced in the hypothalamus, which are judged essential for reproduction in all vertebrates. Within each species, all GnRH forms (Zohar et al., 1995; Parhar et al., 2002) are potent gonadotropin hormone (GtH) releasers, the latter being synthesized and stored in the gonadotropes of the pituitary gland (Hao et al., 1995; Zohar et al., 1995). In general, multiple GtH forms are present in the pituitary of fishes (Holland et al., 1998, 2001).

In these, the hypothalamus lies at the base of the brain in close proximity to the pituitary gland, below it. The first is a neuron-rich structure that synthesizes products transported to the pituitary via a

specialized portal blood supply, in the case of higher vertebrate classes, or via direct neuronal innervation, in the case of osteichthyan fishes (Van Oordt and Peute, 1983; Chow et al., 1998). However, neither a specialized portal system nor direct innervations were found in the agnathan pituitary or in the ventral pituitary lobe of reproductively active chondrichthyan; thus, the assumption of hypothalamic regulation of pituitary function in these fishes is being debated for long (Dodd, 1983; Gorbman, 1983). Therefore, in many but eventually not all fishes, neuronal processes originating in the hypothalamus penetrate into the pituitary, allowing direct neural control of the pituitary function.

Succinctly, the Gomori-positive *nucleus preopticus* (NPO) and the Gomori-negative *nucleus lateralis tuberis* (NLT) are the key components of the fish hypothalamus (Follénius, 1965; Ball and Baker, 1969; Perks, 1969; Sage and Bern, 1971; Peter, 1973; Holmes and Ball, 1974). The NPO comprises the *pars magnocellularis* and the *pars parvocellularis* and it is situated on both sides of the preopticus recess. The axons developing from the NPO cells form the *preopticus* hypophyseal tract, which penetrates the pituitary, ramifies, and terminates in the neurointermediate lobe (Knowles and Vollrath, 1966a, b; Terlou et al., 1978).

The NLT is located in the caudal part of the hypothalamus and is subdivided into the *pars rostralis, pars medialis, pars ventrolateralis,* and *pars lateralis;* the presence or absence of these subdivisions varies amongst different species (Terlou and Ekengren, 1979). For instance, the *pars medialis* and *pars lateralis* were found in the hypothalamus of the mullets *Mugil cephalus* and *M. capito* (Stahl, 1957; Blanc-Livni and Abraham, 1970), whereas all of the above NLT subdivisions were described in the rainbow trout, *Oncorhynchus mykiss* (Follénius, 1963; Terlou and Ekengren, 1979). The axons from the NLT nuclei terminate at the adeno-neurohypophysial interface (Falck et al., 1962). Fibres invade the *rostral* and *proximal pars distalis* of the pituitary, and either innervate the gonadotropes or discharge the secretions into perivascular spaces surrounding these cells (Follénius, 1965; Knowles and Vollrath, 1966a, b; Vollrath, 1967; Leatherland, 1970a, b; Zambrano, 1970a, b; Van Oordt and Ekengren, 1978).

In some species, such as the catfish *Heteropneustes fossilis* (Haider and Sathyasesan, 1972), the NLT secretory products may also be released into blood vessels passing through the neuronal perikarya. In other fishes, e.g., the gobiid fish *Gillichthys mirabilis* (Zambrano, 1970a, b), and the goldfish *Carassius auratus* (Kaul and Vollrath, 1974), fibres similar to those of the

NLT directly innervate the gonadotropes. In other teleosts, the gonadotropes are not directly innervated, as shown for instance in the Atlantic salmon, *Salmo salar* (Friedberg and Ekengren, 1977). Nevertheless, both the NPO and the NLT seem to regulate the gonadotropic functions of the pituitary (Northcutt, 1981). In fact, initial studies performed by Terlou et al. (1978) pointed to a strong correlation among the activity of the NPO with the annual gonadal cycle in the rainbow trout, *O. mykiss;* in this species, the NPO is active during the vitellogenic and spawning periods (June to January), and inactive in the sexually quiescent period (February to May). Similarly, seasonal fluctuations in the quantity of neurosecretory material from the NPO have been correlated with the gonadal activity in catfish, *H. fossilis* (Viswanathan and Sundararaj, 1974a, b).

The pituitary gland is composed of the adenohypophysis, derived from the embryonic Rathke's pouch, and the neurohypophysis, originating from the diencephalon. The adenohypophysis, which regulates gonadal functions in fishes, is the site of synthesis, storage and release into circulation of several peptide and protein hormones. The adenohypophysis is divided in pro-adenohypophysis (or rostral *pars distalis*), meso-adenohypophysis (or proximal *pars distalis*), and the meta-adenohypophysis (or *pars intermedia*) (Ball and Baker, 1969; Schreibman et al., 1973). This gland controls reproduction via a dual GtH system: GtH-I and GtH-II, these being produced in two (immunocytochemically) distinct types of gonadotropes, named FSH (GtH-I) cells and LH (GtH-II) cells (Shimizu et al, 2003). The duality of the gonadotropins has been shown in a number of teleosts, such as coho salmon, *Oncorhynchus kisutch* (Swanson et al., 1991); rainbow trout, *O. mykiss* (Schulz et al., 1992); carp, *Cyprinus carpio* (Van der Kraak et al., 1992); bonito, *Katsuwonus pelamis* (Koide et al., 1993); and killifish, *Fundulus heteroclitus* (Lin et al., 1992).

Changes in the concentrations of hypothalamic GnRH in the sea lamprey, *Petromyzon marinus,* and goldfish, *C. auratus*, are correlated with the release of GtHs, by the pituitary, and with gonadal recrudescence (increased gametogenic activity) (Fahien and Sower, 1990; Yu et al., 1997). Meanwhile, the current notion that in all vertebrates multiple forms of GnRH coexist in the brain of individual species (King and Millar, 1995) indicates that, during evolution, GnRHs may have acquired additional functions that are not necessarily related to reproduction. More

recently, it was advanced that fish possess two or three variants of GnRH, whereas at least 14 variants have been identified from various vertebrates (Gothilf et al., 1996; Holland et al., 2001). The phyletic distribution of GnRH forms in fishes and other vertebrates thus provides an interesting perspective on the evolution of hormones.

Teleost fishes of the Perciform Order are the first group of vertebrates in which three distinct forms of GnRH ($GnRH_{salmon}$, $GnRH_{chicken}$, and $GnRH_{seabream}$) have been conclusively demonstrated to co-exist within a single species, as shown first in the gilthead seabream, *Sparus aurata* (Powell et al., 1994). Subsequently the presence of three distinct forms of GnRH was confirmed in the brain of other perciform species, e.g., in the astatotilapia, *Haplochromis burtoni* (White et al., 1995, 2002), the striped bass, *Morone saxatilis* (Hassin et al., 1998), and the tilapia, *Oreochromis mossambicus* (Weber et al., 1997). More recent studies in other species conducted to similar conclusions (Nabissi et al., 2000; Somoza et al., 2002; Yamamoto, 2003). Each of the three forms of the GnRH has a restricted regional pattern in the brain (White et al., 1995), and appears to be regulated by both hormones and environmental cues. Recently, a seasonal variance of the expression of genes encoding these three GnRHs were reported in red seabream, *Pagrus major* (Okuzawa et al., 2003).

For the coho salmon, *O. kisutch*, it was suggested that GtH-I is involved in regulating gonadal steroidogenesis from puberty to early gonadal development, whereas GtH-II is involved in regulating the final stages of reproductive maturation and spawning (Swanson et al., 1991); a scheme that is likely to be common in teleosts (Figs. 18.1, 18.2). Recent studies revealed that both GtHs can either act directly on the GnRH receptors in the hypothalamus, where they are able to modify the GnRH release, or key may act at the level of the gonads, where they control the synthesis and release of the steroid hormones involved in fish reproduction (Peter and Yu, 1997; Evans, 1999; Wang and Ge, 2003).

In the agnathans, which encloses most primitive vertebrates, two GnRH neurohormones are involved in the pituitary-reproductive activity of the sea lamprey, *P. marinus* ($GnRH\text{-}I_{lamprey}$ and $GnRH\text{-}III_{lamprey}$) (Sherwood et al., 1986; Sower et al., 1993). More recent studies indicate that the latter GnRH forms are also present in the other two families of lampreys, *Geotriidae* and *Mordaciidae* (Sower and Kawauchi, 2001). In chondrichthyans, two GnRH neurohormones were described for the elasmobranch dogfish *Squalus acanthias* ($GnRH_{dogfish}$ and $GnRH\text{-}II_{chicken}$)

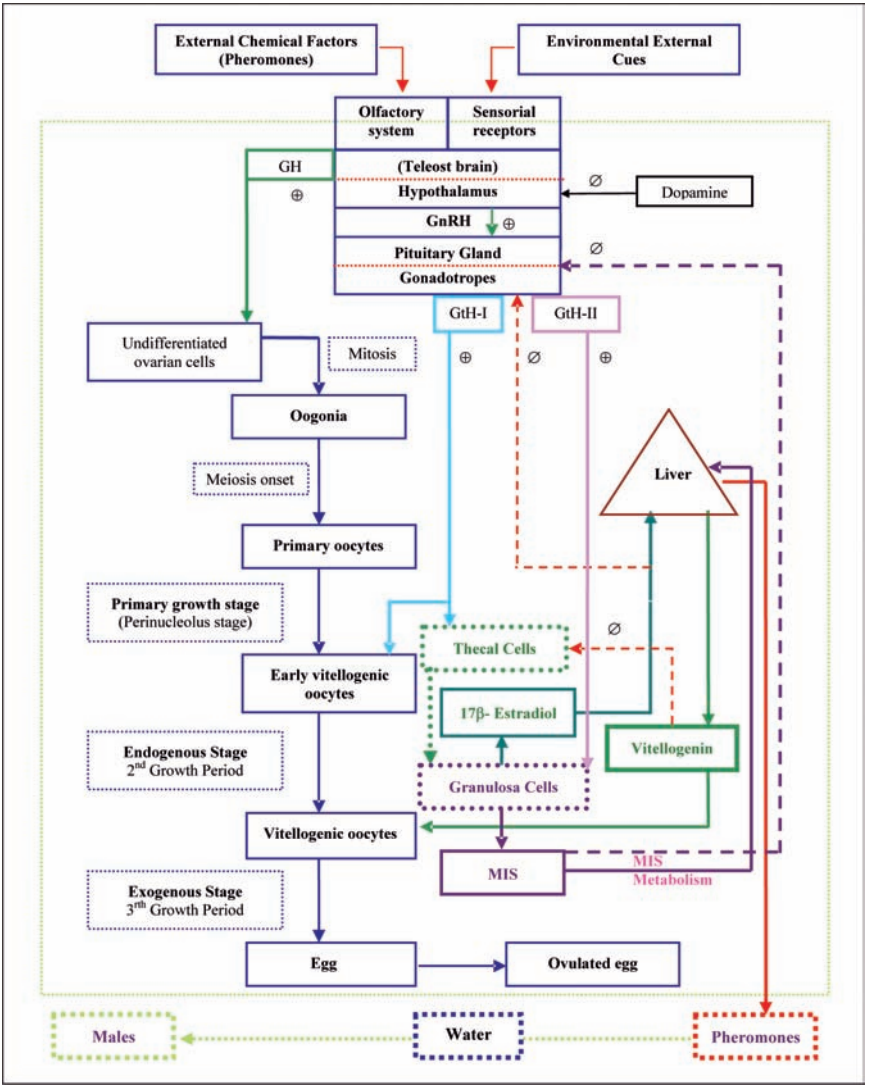


Fig. 18.1 General aspects of reproduction control in females. External stimuli influence the brain to release specific neurohormones and neurotransmitters. Consequently, secretion rates of hypothalamic hormones and neuromodulators (namely GnRHs and dopamine), of pituitary gonadotropins (GtH I and GtH II), and of sexual hormones, including the maturating inducing steroids (MIS), all vary during the reproductive cycle. The symbols ø and ⊕ represent, respectively, inhibition and induction.

(Lovejoy et al., 1992), and one form was sequenced for one holocephalan, the ratfish *Hydrolagus colliei* (GnRH-$II_{chicken}$) (Lovejoy et al., 1991).

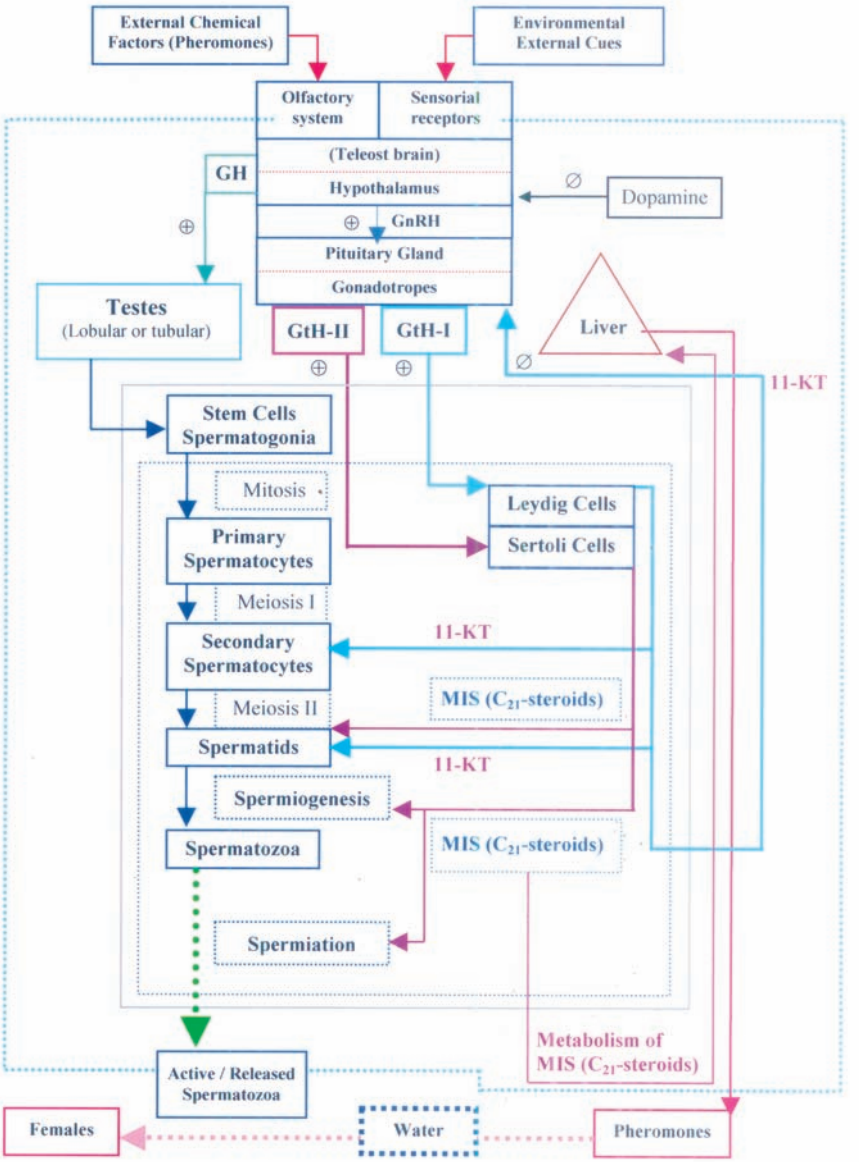


Fig. 18.2 General aspects of reproduction control in males. Like in females, the external stimuli impinge the brain to release specific neurohormones and neurotransmitters. Consequently, the secretion rates of hypothalamic hormones (GnRHs or dopamine), of pituitary gonadotropins (GtH-I and GtH-II), and of sexual hormones, all vary during the reproductive cycle. Irrespective of testicular types, sexual maturation involves the same precursors. The symbols ø and ⊕ represent, respectively, inhibition and induction. 11-KT (11-ketotestosterone); GH (Growth Hormone); MIS (Maturation Inducing Steroid).

Apart from GnRH, the major regulator of gonadotropes in mammals, there are also targets for a number of other hypothalamic factors, such as pituitary adenylate cyclase-activating polypeptide (PACAP), neuropeptide-Y (NPY), galanin, endothelin, oxytocin, vasoactive intestinal polypeptide (VIP), and also substance-P (reviewed by Evans, 1999). These peptides participate in the regulation of gonadotropins. In goldfish, *C. auratus*, NPY was found to increase the GtH-II release by acting directly on the pituitary or by increasing GnRH release (Peng et al., 1993). The PACAP is a member of the glucagon/secretin peptide family, and its molecular structure is highly conserved in vertebrates. The functional role of PACAP in regulating the growth hormone (GH) was reported to act as a growth hormone releasing hormone (GHRH) in the European eel, *Anguilla anguilla* (Montero et al., 1998), and in the goldfish, *C. auratus* (Wong et al., 1998, 2000); where it was also found to be an effective stimulant of GtH-II release, both *in vivo* and *in vitro*. By the contrary, dopamine (DA) acts as a GtH-II release inhibitory factor in a wide range of teleosts, and has been exhaustively reviewed (Peter, 1986). DA directly inhibits basal, as well as GnRH-stimulated GtH-II release (Peter, 1986; Trudeau et al., 1996). Several studies have confirmed that removal of dopaminergic inhibition, together with increased GnRH stimulation, constitute a very important neuroendocrine mechanism that leads to the preovulatory GtH-II surge and ovulation in many species (Peter et al., 1991). However, the Atlantic croaker, *Micropogonias undulatus*, is an exception example, in the sense that no evidence of DA inhibition of GtH secretion was found (Copeland and Thomas, 1989 a, b). Later, it was cited that, in this species, γ-aminobutyric acid (GABA) is responsible for the stimulatory and inhibitory influences on GtH-II secretion (Khan and Thomas, 1999). Recently, Mathews et al. (2002) suggested that the last process is controlled by a negative feedback system promoted by the maturation-inducing steroids (MIS).

Gonadotropic hormones (GtH-I and GtH-II) and the thyroid-stimulating hormone (TSH) are glycoproteins, closely related to each other, that are produced and secreted by the pituitary and act primarily at the level of the gonad and thyroid, respectively. All three hormones consist of a α subunit and a β subunit. The αβ dimmer forms after transcription to become the active hormone. The α subunit is common to all three hormones; specificity is conferred by the β subunit. The actions of GtHs on gonadal tissue are mediated by specific membrane-bound receptors. Analysis of GtHs receptors in fishes, namely at molecular level, is a field generating great interest, with advances being well illustrated by

the works of Bogerd et al. (2001), Kumar et al. (2001a, b), and Wang and Ge (2003), among other. Quite recently, cloning and characterization of zebrafish, *Brachydonio rerio,* GtH-I (FSH) and GtH-II (LH) receptors evidenced that they have different roles in ovarian follicle development; moreover, it was shown that in addition to their abundant expression in the gonads, they can also be found in both liver and kidney (Kwok et al., 2005).

ENVIRONMENTAL CUES AND HISTOPHYSIOLOGY OF THE GONADS

Environmental factors have long been considered to play an important role in teleost reproduction. In the past, many studies referred that photoperiod, rainfall, floods, water temperature, dilution of electrolytes, oxygen content, pH, and lunar cycles, all affect reproduction in fishes (Liley, 1976; Billard and Breton, 1978; Schwassman, 1978; Broton et al., 1980). Presently, this theme is an important topic of study given that a great number of species and habitats are yet to be studied and others are being disturbed by diverse factors (Eastman and De Vries, 2000; Murua and Motos, 2000; Rahaman et al., 2000a, b; Harahap et al., 2001, 2002; Paugy, 2002; Weltzien et al., 2002).

Given the aforesaid information, we provide shall now brief integrative considerations about the role played by the environmental stimuli over the gonadal histophysiology.

We have known for long that fishes interplay their physiological functions with environmental cycles and that endogenous periodicities of physiological processes are responsible in part for seasonal reproduction (Bullough, 1939; Sundararaj and Vasal, 1976; Sundararaj et al., 1980). In fact, certain proximate environmental factors acting as signs for the approaching favourable season for reproduction are perceived by the diverse exteroceptors, and through them affect the central nervous system, the pituitary and, finally, the gonads (Figs. 18.1, 18.2). It is via such environmental factors that the endogenous rhythm is brought into phase for the precise breeding time. Therefore, it is not surprising that the majority of fishes are seasonal breeders; however, a few breed continuously or, by the contrary, only once during their lifetime.

General Aspects of Ovary Histophysiology

A basic similarity in the organization of the gonads occurs throughout the vertebrates. Naturally, there are a lot of peculiarities within each

taxonomic group. Hoar (1969) wrote a crucial dissertation on gonadal embryology, anatomy, and phylogeny in fishes. Subsequent detailed reviews were written for agnathan (Gorbman, 1983), chondrichthyan (Dodd, 1983) and teleostean (Nagahama, 1983).

In teleosts, the ovaries of adults generally are paired structures attached to the body cavity on either side of the dorsal mesentery; Nagahama (1983) described exceptions to this rule. Oviducts occur in most teleosts; however, they are absent in lampreys (Petromyzontidae) and hagfishes (Myxinidae), and lost secondarily during development in certain elasmobranchs and teleosts (Hoar, 1969).

A global understanding of the connection between environment and the physiologic development of the gonads included the knowledge of the developing ovarian follicle structure as well as of the ovary histological pattern. As to the first point, the organization of the emergent ovarian follicle is rather similar amongst different fish species. Follicle cells bound the generally centred developing oocyte, forming a characteristic cellular complex, irrespective of cytological differences in details.

The follicle cell layer usually consists of an inner well-defined stratum, the granulosa cell layer, and one or two outer sublayers of theca cells. Once the oocyte starts growing, the follicular layers change, in order to support, nourish, and regulate its development in a continuous manner (Nagahama, 1994). The pituitary initially controls the release of GtH-I, which stimulates the production of sexual hormones by the theca cells, such as testosterone (T), and its aromatization to 17β-estradiol (E_2) in the granulosa (Nagahama, 1994; Peter and Yu, 1997). In a prompt response to the E_2 stimulus, the liver produces vitellogenin, which is sequestered by the oocytes in a receptor-mediated process enhanced by GtH-I. Details of this mechanism can be found in selected books (Ng and Idler, 1983; Wallace, 1985; Nath, 1999), and in the recent open access review by Arukwe and Goksøyr (2003). Androgens and GH can also induce hepatic vitellogenesis, probably as a result of their conversion to estrogens by the hepatic aromatase (Peyon et al., 1993, 1996).

Meiotic maturation of fish oocytes is induced by the action of the MIS, which are C_{21}-steroid hormones. The 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and the 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P) are the most important MIS, identified in many teleost species (Ng and Idler, 1983; Theofan, 1983; Nagahama and Adachi, 1985; Wallace, 1985; Yaron, 1995; Rocha and Reis-Henriques, 1996, 1999; Nath, 1999). The interaction of the two ovarian follicle cell layers (i.e.,

theca and granulosa) is required for the synthesis of the MIS. The more external theca layer produces 17α-hydroxyprogesterone (17-P) that is converted to MIS in the underneath granulosa cells, by the action of 20β-hydroxysteroid dehydrogenase (20β-HSD) (Kanamori et al., 1988). The preovulatory surge of GTH-II is responsible for the rapid expression of 20β-HSD mRNA transcripts in the granulosa cells during oocyte maturation (Kanamori et al., 1988). In addition, the presence of the MIS also induces oocyte maturation by acting on a pertussis toxin-sensitive G-protein-coupled membrane receptor (Yoshikuni and Nagahama, 1994). Actually, the early steps of the MIS action involve the creation in the oocyte of a downstream mediator of the maturating C_{21}-steroid hormone: the maturation-promoting factor or metaphase-promoting factor (MPF). The MPF consists in the cdc2 kinase and the cyclin B (Yoshikuni and Nagahama, 1994). Upon egg activation, the MPF is inactivated by degradation of cyclin B (see Thomas et al., 2003) for further information on the nature and identity of progestin and estrogen membrane receptors in fish gonads).

Correlations between environmental conditions and the gonadal ripeness have been evaluated by employing either macroscopic parameters (e.g., Buñag, 1956; El Zarka, 1962), or simple oocyte size classification (e.g., Siddiqui, 1979; Dadzie and Wangila, 1980; Jalabert and Zohar, 1982), or even by subdivision of follicles into discrete developmental stages based upon histological appearance (e.g., Avarindan and Padmanabhan, 1972; Dadzie, 1974; Hussein, 1984). West (1990) discussed the relative merits and disadvantages of the methods available at the time. Facing the technical advances since then, it is urgent that the new generation of stereological tools for evaluating oocyte and follicular size and number (e.g., Calado et al., 2001, 2003) substitute in fish studies the still-used but outdated approaches.

For the histological grading of oocyte (and follicle) maturation several criteria have been in use: size, amount and distribution of cell inclusions (specially yolk granules), and chromosome plus chromatin morphology. Table 1 includes the various stages of oocyte development, using as a model the ovary of tilapia, *Oreochromis mossambicus*; each stage being defined by oocyte size, appearance of both nucleus and nucleolus, the type of cytoplasmic inclusions, and developmental degree of the enveloping layers.

Oogenesis always begins with the differentiation of germ cells, which produce gametes, and somatic cells, which will later differentiate into follicular layers. It is debated whether true oogonial stem cells remain in

adult fish, despite definitive formal proofs are to be provided yet. Briefly, stem cells proliferate and undergo changes that turn them into oogonia (Table 18.1: A). Then, meiosis starts, but the cell 'freezes' at the diplotene stage of the first meiotic division, and the former oogonia turn into primary oocytes (Nagahama, 1983). These start to enlarge, undergoing what is usually called primary growth.

The primary growth stage is sometimes divided in two phases: the chromatin nuclear and the perinucleolar stages (Table 18.1: B, C). Primary growth is characterized by a substantial increase of the cell size and by the formation of the Balbiani bodies, also called 'yolk nucleus'. These appear at light microscopic level as basophilic cytoplasmic masses, corresponding to various cellular organelles, such as Golgi apparatus, endoplasmatic reticulum cisternae, multivesicular bodies, and even lipid granules (Beams and Kessel, 1973; Guraya, 1979; Wallace and Selman, 1981).

The secondary growth period begins when prominent vesicles appear, firstly in the outer and midcortical areas of the cell, steadily increasing their number and size, until occupying almost the entire ooplasm. This process is known as 'cortical alveoli stage' or 'endogenous vitellogenesis' (Table 18.1: D, E), and the vesicles named as 'cortical alveoli', despite coined also as 'yolk vesicles', 'primary yolk' or 'endogenous yolk'; this because they are rich in glycoproteins produced by the oocyte itself. Since the 'cortical alveoli' release their content upon fertilization, they are considered homologous to the 'cortical vesicles' or 'cortical granules' of other vertebrates (Selman and Wallace, 1989; Wallace and Selman, 1990). Nevertheless, these authors strongly suggested that such terms including 'yolk' should not be used 'since the cortical alveoli do not provide nutrients for the developing embryo'. During the secondary growth stage, the follicular layers differentiate, displaying both the theca and granulosa layers, which are separated from each other by a basement membrane (Guraya, 1986, 1996). In addition, between the surface of the oocyte and the granulosa cell layer an acellular layer, zona radiata, develops, appearing at light microscopy as an eosinophilic tranluscid band; this is considered to be still immature at stage (Guraya, 1986, 1996).

The tertiary growth period, greatly controlled by the pituitary GtH-II, is known as the exogenous vitellogenesis. During this period (Table 18.1: F-J), the enlargement of the oocyte is attributed mainly to the accumulation of true yolk, being the yolk precursor integrated into the oocyte by micropinocytosis (Droller and Roth, 1966; Shackley and King,

Table 18.1 Summary of oocyte developmental stages in tilapia, *O. mossambicus*. Haematoxylin-eosin staining.

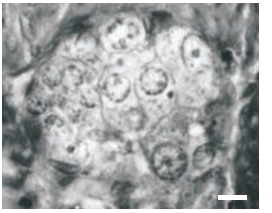
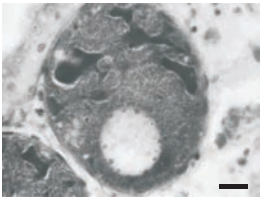
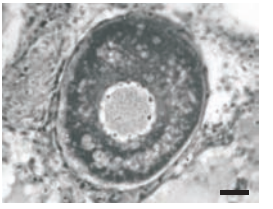
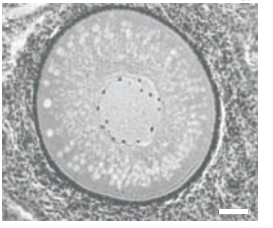
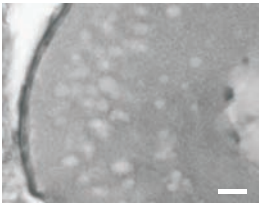
Oocyte developmental stage	*Main cellular characteristics*	*Histological images*
Oogonium stage		A
Proliferating oogonia	Appear as small nests of mitotic cells dispersed within the connective tissue. Ooogonia are round, small (Ø ≈ 5-15 μm), with scarce and clear cytoplasm. Typically, they have one nucleolus. Bar (A) = 8 μm.	
Primary growth		B
Early perinucleolus stage	Primary oocytes (Ø ≈ 90 μm) showing one large peripheral nucleus, containing large basophilic nucleoli. The cytoplasm is also basophilic and dense structures, known as Balbiani bodies, begin to appear. Follicular cells are not observed. Bar (B) = 13 μm.	
		C
Advanced perinucleolus stage	The oocyte becomes a larger cell (Ø ≈ 150 μm) with a more centrally located nucleus, containing several nucleoli. The cytoplasm is strongly basophilic and the Balbiani bodies are spread throughout the cytoplasm. During this stage, the follicular layers are still undifferentiated. Bar(C) = 25 μm.	
Secondary growth		D
Early vitellogenesis or Endogenous vitellogenesis or Cortical alveoli stage or Yolk vesicle stage	Relatively to the previous stage, the oocyte (D) more than duplicates its size (Ø ≈ 300 μm). The cytoplasm becomes acidophilic and shows many evident and accumulating vesicles, often called endogenous yolk. Lipid droplets also spread all through the cytoplasm. The follicular layers begin to differentiate (E). Bar (D) = 40 μm. Bar (E) = 11 μm.	 E 

Table Contd.

Table Contd.

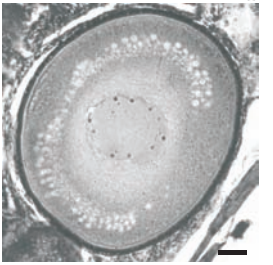
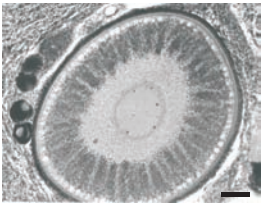
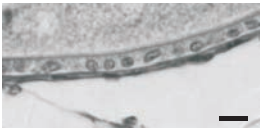
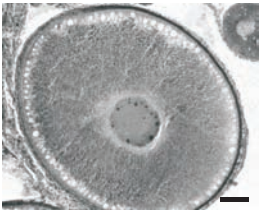
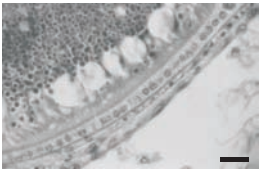
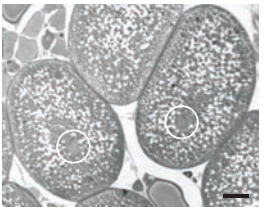
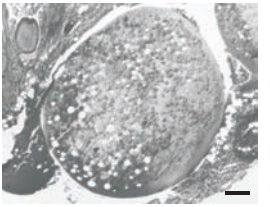
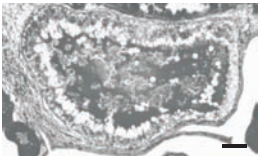
Oocyte developmental stage	*Main cellular characteristics*	*Histological images*
Tertiary growth	The oocyte continues to enlarge (Ø ≈ 400 μm). Comparatively with the former stage, the cytoplasm is accentually acidophilic and the endogenous yolk (cortical vesicles) starts migrating to the periphery (F). Bar (F) = 45 μm.	**F** 
Exogenous Vitellogenesis I	During this stage, the morphological aspect of the oocyte changes whereas the intake of exogenous yolk takes place (G). At this point, the lipid droplets are also pushed to the periphery of the oocyte, whereas the forming yolk granules became located in a kind of radial formation. Bar (G) = 135 μm.	**G** 
	The follicular layers (H)—theca (external and flattened) and granulosa (internal, with cubic cells)—are well developed and easily perceived. Bar (H) = 10 μm.	**H** 
Tertiary growth	The oocyte continues to grow (Ø ≈ 1000 mm) but the texture of the cytoplasm modifies. It becomes denser, even more acidophilic, and the distribution of yolk seams homogeneous. The vesicles and oil droplets do accumulate all together, along the periphery of the oocyte. Bar (I) = 130 μm.	**I** 
Exogenous Vitellogenesis II	The follicular layers (J) are so developed that can be easily seen even at low magnification. From the inner side we can see: zona radiata, granulosa layer (cubic cells), and the flattened theca. Bar (J) = 15 μm.	**J** 

Table Contd.

Table Contd.

Oocyte developmental stage	*Main cellular characteristics*	*Histological images*
Final growth period		**K**
Migratory nucleus stage	The oocyte grows and achieves its maximum dimension (Ø ≈ 1500 μm). The nucleus (circle) begins its migration towards one pole and later desintegrates. The yolk granules became more compact and start fusing. Lipid droplets can be found amidst the merging yolk granules. Bar (K) = 200 μm.	
Ripe stage		**L**
Ovulation	The mature tilapia oocyte becomes an extremely compact cell. As a rule, fish egg hydration precedes the release of the mature oocyte into the oviduct. Bar (L) = 200 μm.	
Atresia		**M**
Early atresia	Atretic follicles are characterized by moderate or severe structural distortion, desorganization, and cell degeneration. Atresia can occur at any developmental stage. Ultimately, phagocytic action of diverse nature cleans up the debris. Bar (M) = 70 μm.	

1977; Patiño et al., 2000). The yolk precursor proteins are synthesized in the liver, and freed into the blood in response to E_2 released by the granulosa cells, mainly after conversion from testosterone produced by theca cells (Fig. 18.1). During this phase both theca and granulosa cells became very active, and their size drastically increase. The glycoprotein rich zona radiata thickens, and microvilli, originating from both the oocyte and granulosa cells, penetrate that zona through the so-called 'pore channels'. Additionally, gap junctions between granulosa cells and the oocyte have been identified (Guraya, 1996). Those junctions seem to help forming an efficient metabolic syncytium between granulosa cells and the oocyte.

Throughout the final growth period (Table 18.1: K), the oocytes tend to lose their spherical shape and become slightly flattened. In the animal

pole, on one of the compressed surfaces, and located around a small depression in the follicle, the tunnel-shaped micropyle develops through the zona radiata. During this period, the MIS are largely produced, especially in granulosa cells (Nagahama et al., 1995). At this stage, the first meiotic division resumes and the oocytes became the denominated eggs (Fig. 18.1). Finally, the follicular layers start collapsing and the ovulation takes place (Table 18.1: L). Post-ovulatory remains are left in the ovary. Morphological aspects of the final oocyte maturation (FOM) vary a little among species, but can be generally characterized by the lipid coalescence, migration of the nucleus to the oocyte periphery, breakdown of the nuclear envelope, yolk coalescence and, finally, hydration. Importantly, the ovulated egg (released from the ovary wall) continues the meiosis, upto the second meiotic metaphase, the point at which fertilization becomes possible; sperm binding triggers the completion of meiosis.

Atresia (degeneration) of oocytes is a common event in the fish ovary. Follicles can start to be atretic at any stage of development (Table 18.1: M), passing through several morphological stages until the complete resorption of the cellular remnants (Nagahama, 1983; Miranda et al., 1999). Initial resorption (during the intermediate and advanced periods of atresia) seems to be primarily made by follicle cells, with both granulocytes and macrophages intervening at the final stages of 'clean up', in which yellow-brownish pigments can be found. Little is known about the cellular and especially molecular mechanism involved in follicular atresia, although it does involve apoptosis triggering and hormonal modelling (Jans and Van Der Kraak, 1997). Comparing atresia in species with dissimilar types of oocyte synchrony would be valuable in understanding how the process is governed under the different physiological scenarios.

Synchrony and Asynchrony of Ovarian Maturation

Considering the physiologic variations in fish, four main patterns of ovarian development are generally seen, and these are related with their reproductive strategies (Wallace and Selman, 1981; Tyler and Sumpter, 1996): synchronous, group-synchronous, multiple-batch group-synchronous and asynchronous.

In short, in a synchronous ovary all oocytes, once formed, grow and ovulate from the ovary in unison (of course, expect for those that became atretic). Further replacement of one stage by an earlier stage does not take

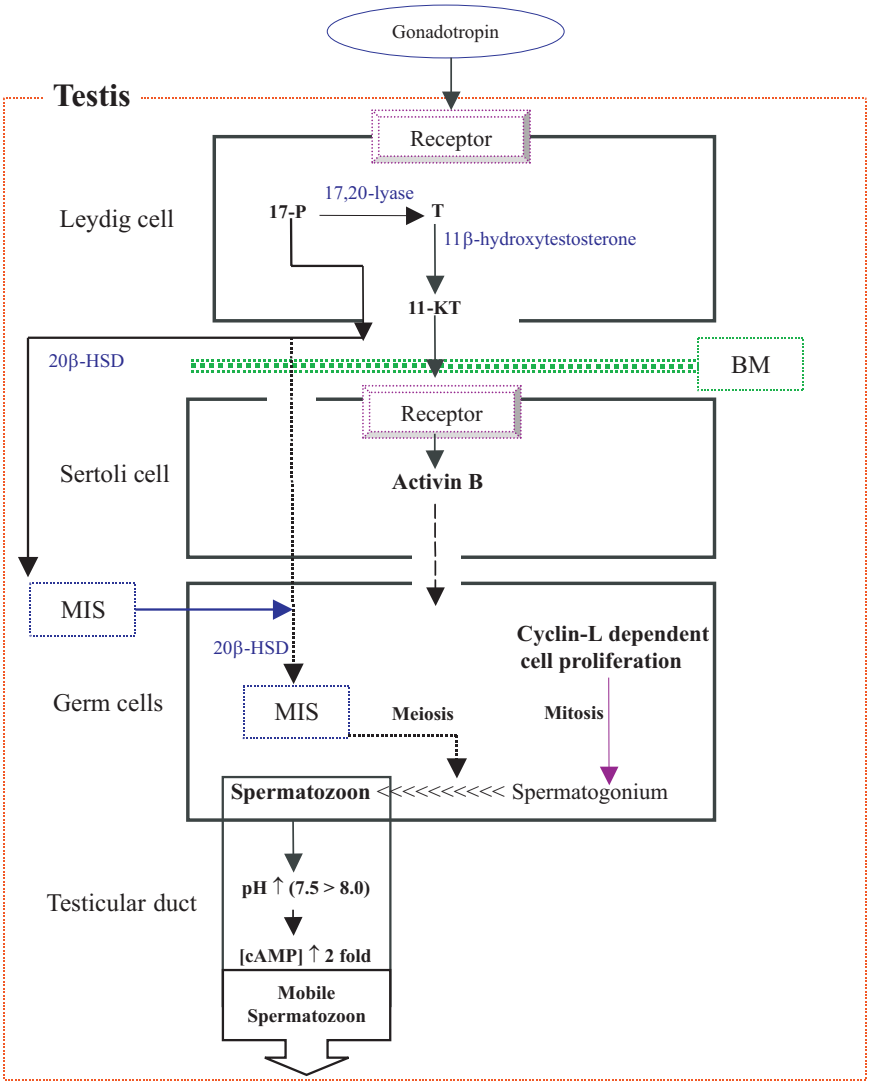


Fig. 18.3 Particular aspects of reproduction control in males. 11-KT (11-ketotestosterone); 17-P (17α-hydroxyprogesterone); 20β-HSD (20β-hydroxysteroid dehydrogenase); BM (Basal Membrane); cAMP (cyclic AMP); MIS (Maturation Inducing Steroid). The range of values for both pH and [cAMP] are based on acquirement of sperm motility in salmonids (Yaron, 1995).

place. Such ovaries may be found in teleosts that spawn once and then die, such as the pacific salmons, *Oncorhynchus* spp., the lampreys (Petromyzontidae) and the freshwater eels, *Anguilla anguilla*. These fishes

have a short, well-defined spawning season, and synchronous follicular development during which the levels of E_2 and T peaked at, or near the beginning of ovulation (Kime, 1993). At the completion of vitellogenesis, a surge in plasma GtH-II stimulates a drop in plasma E_2. A transient increase in plasma T during GV migration, and a striking elevation in the plasma levels of the MIS, which acts at the level of the oocyte membrane, induces FOM (Nagahama, 1994; Nagahama et al., 1994; Peter and Yu, 1997). GtH-II causes final oocyte maturation by inducing ovarian maturational competence and by stimulating the follicle cells to synthesize the MIS (Nagahama et al., 1995). Studies in a variety of teleosts with synchronous (or, to some extent, synchronous) ovarian development show that in these species the most potent steroid inducing oocyte final maturation is the 17,20β-P (Nagahama et al., 1983, 1995; Theofan and Goetz, 1983; Nagahama, 1987; Yaron, 1995).

Fish with a group-synchronous ovary are seasonal breeders, in general, and at least two populations of oocytes can be distinguished at some time (Fig. 18.4); a moderately synchronous population of large oocytes (defined as a 'clutch') and a more diverse population of smaller oocytes from which the clutch is recruited. This is by far the most common situation among teleosts (Wallace and Selman, 1981), irrespective of the wide variation in the time of the year when breeding occurs. We introduce in the following paragraphs a few relevant illustrative examples of how different this breeding pattern can be.

Group-synchrony can be found in deep-water and cold-water fish species, such as the common trouts, where spawning occurs in a short period of the year. In fact, in cold/deep waters, factors like water temperature, profuse food, corporal length and oocyte depots of lipids and vitellogenin are determinants for successful spawn. This was derived from studies made in species living in such cold habitats (e.g., Eastman, 1993). More recently, such determinants were further confirmed for the Antarctic toothfish, *Dissostichus mawsoni* (Eastman et al., 2000), the roughed grenadier, *Macrocourus berglax*, (Murua and Motos, 2000), the Atlantic halibut, *Hippoglossus hippoglossus* (Weltzien et al., 2002), and the swordfish from the Northwestern Atlantic, *Xiphias gladius* (Arocha, 2002).

In temperate regions, factors such as photoperiod, water temperatures, and profuse food are the main determinants for successful spawn. In general, fishes living in these habitats have a multiple-batch group-synchronous ovary, and are extended seasonal spawners that undergo multiple ovulations within the course of a few days or weeks, as

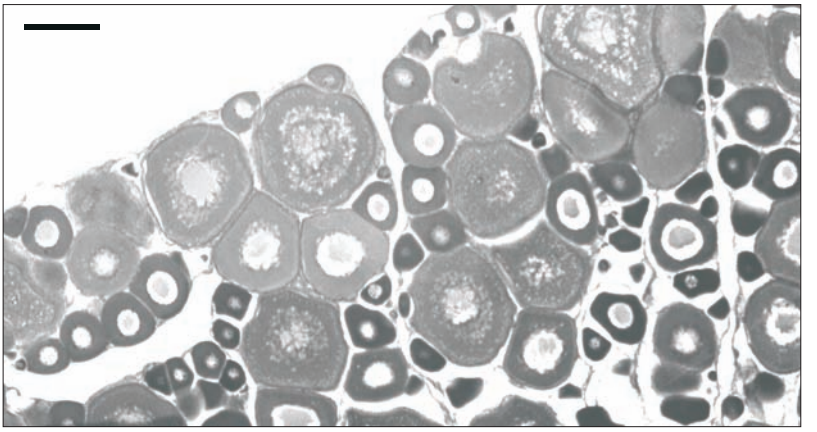


Fig. 18.4 General histological aspect of the ovary of sea bass, *D. labrax*. Most of the oocytes are either in primary or in tertiary growth stages. Haematoxylin-eosin staining. Bar = 335 μm.

seen in the European sea bass, *Dicentrarchus labrax* (Fig. 18.4) (Rocha and Reis-Henriques, 1999, 2000). These latter studies revealed that the active follicles, able to produce either E_2 or MIS, are not ripe at the same time. Therefore, during the all year, including the spawning period, the circulating levels of all sexual hormones are low (Fig. 18.5) when compared with species showing more synchronous patterns of ovary development.

In tropical waters, seasonal rainfall, water quality, and food availability are the main determinants for successful spawn, as shown during the years for diverse species (Mc Kaye, 1977; Kramer, 1978; Munro, 1990; de Silva, 1991; Harikumar et al., 1994; Lévêque, 1997; Lévêque and Paugy, 1999; Paugy, 2002). In central Amazon, freshwater fishes spawn during the rainy season (Schwassmann, 1978). In Africa, recent studies demonstrated an influence of such environmental conditions on the reproductive strategies of tropical fishes (Lévêque, 1997; Lévêque and Paugy, 1999; Paugy, 2002). The latest factors are also important for the Indian subcontinent and Malaysia. In these habitats, the vast majority of the freshwater fishes show multiple group-synchrony and breed during the monsoon season, when rainfall is heaviest (Jhingran, 1991; McAdam et al., 1999).

In general, the majority of fishes living in tropical environments have multiple-batch group synchronous ovaries or asynchronous ovaries. In the latter situation, oocytes of all stages are present without dominant

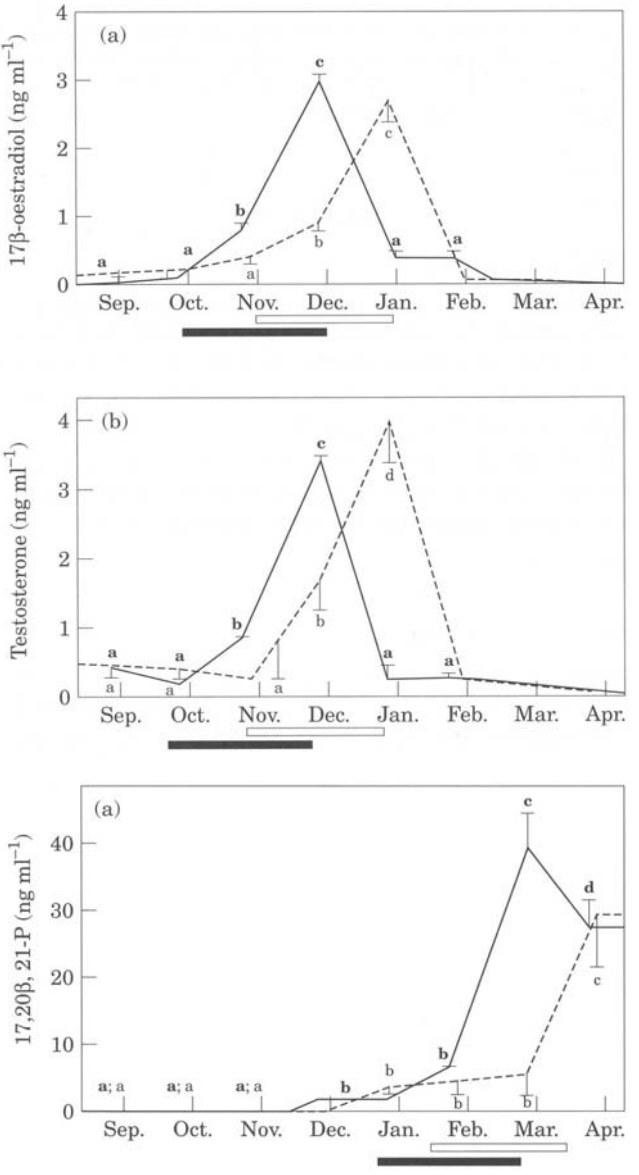


Fig. 18.5 Seasonal changes in plasma (mean ± SE) of 17β-oestradiol, testosterone and 17,20β,21-P in both captive (n=10; broken line in the graphs) and feral (n=5; continuous line in the graphs) sea bass, *D. labrax*. Horizontal bars at the bottom of the graphs indicate the duration of the gametogenic period of captive (o) or feral (n) females. Points designated by different letters (bold letters– feral fish) are significantly different from each other under a statistical analysis (Rocha and Reis-Henriques, 1999 - Reproduced with the kind permission of Blackwell Publishing).

populations being seen in the gonad (Wallace and Selman, 1981). This state appears to apply to those teleosts that have a population of primary oocytes and heterogeneous populations of vitellogenic oocytes, from which several batches are recruited, and undergo maturation during the annual spawning season, in regular or semi regular intervals. Examples are the killifishes, *Fundulus* spp., and the tilapia, *Oreochromis* spp. As such, it is sometimes difficult to differentiate between the two first-cited modes of ovarian development, especially in species with a moderate spawning frequency (i.e., three to five times per year). The distinction between the multiple-batch group-synchronous (Fig. 18.4) from the asynchronous ovaries (Fig. 18.6) can be based on the occurrence; in the latter case, of diverse populations of oocytes undergoing development in a synchronous fashion.

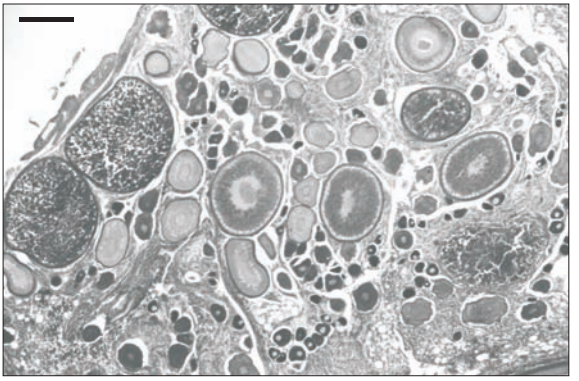


Fig. 18.6 General histological aspect of the ovary of tilapia, *O. mossambicus*. Oocytes in the most diverse stages of development can be found, from primary to final growth stages (see Table 1 for staging details). Haematoxylin-eosin staining. Bar = 335 μm.

Given that the ripeness of the ovarian follicles involves the synthesis and release of sexual steroid hormones, it is understandable that their fluctuation patterns are typical for each group of fishes showing the same type of ovarian development. When compared to synchronous and group-synchronous species, both the multi-batch group-synchronous species and especially the asynchronous species have few follicles able to produce the maturative steroids, which are active in the gonads only for a brief period before metabolization (Scott and Canario, 1992; Rocha and Reis-Henriques, 1998, 2000); the latter occurring mainly in the liver by an A-ring reduction, either by sulphate or glucuronide conjugation (Scott and

Canario, 1992). Thus, even when asynchronous species are sexually ripe and ready to spawn their plasma levels appear to be almost constant (Rocha and Reis-Henriques, 1996, 1998). This attribute is vital for the maintenance of the follicles with other stages of development (Pankhurst and Carragher, 1992; Kime, 1993; Rocha and Reis-Henriques, 1998, 2000). In species such as the tilapia, *O. mossambicus*, there is no decline in E_2 levels prior to oocyte maturation, and T appears high in these mouthbreeders until the latter half of the brooding period (MacGregor et al., 1981; Smith and Haley, 1988; Rocha and Reis-Henriques, 1996, 1998). Testosterone levels fall only upon the cessation of the mouthbrooding behaviour (MacGregor et al., 1981). Multiple-batch group-synchronous species do change their steroid plasma levels with the breeding cycle, but at a much lower degree when compared with the more synchronous species (Rocha and Reis-Henriques, 1999, 2000).

Beyond photoperiod, existence of nutrients, and water conditions, the lunar periodicity is a determinant factor in reproduction of certain groups of marine teleosts. In fact, lunar- or semi lunar-synchronized spawning cycles have been characterized for large groups of fishes; for instance in siganid species, like the rabbitfish, *Siganus guttatus* (Hara et al., 1986; Rahman et al., 2000a, b; Harahap et al., 2001), and the spiny rabbitfish, *S. spinus* (Harahap et al., 2001). Similar behaviour has also been described for the killifishes (*Fundulus* spp.), e.g., *F. similis* and *F. grandis* (Greeley et al., 1986, 1988), and the honeycomb grouper, *Epinephelus merra* (Lee et al., 2002). This reproductive strategy promotes synchronization of gamete release, which favours the dispersal of fertilized eggs and subsequent development and survival of larvae (Garcia, 1992). Thus, it is considered that lunar (and/or tidal) rhythms synchronize the endocrine organs and induce initiation of gonadal maturation and spawning.

The evaluation of ovarian growth in teleosts has been undertaken in diverse ways, from the simple ovary weight relative to body weight ratio to the more troublesome digestion of ovarian tissue in mercury-based solutions (usually Gilson's fluid), followed by determination of oocyte size distribution (Srisakultiew, 1993; Tyler et al., 1996). Other quantitative evaluations used the simple but very reliable stereological point-counting methods (Bromage and Cumaranatunga, 1988; Smith and Haley, 1988; Prat et al., 1990; Tacon et al., 1996). Stereology has also proved valuable to the estimation of fecundity in fishes (Coward and Bromage, 1988, 2001, 2002; Isaac-Nahum et al., 1988; Emerson et. al., 1990; Kestemont, 1990; Srisakultiew, 1993; Macchi et. al., 1995). The obvious advantage of using

quantitative morphology is to translate structure into numbers so to be able to correlate them with functional evaluations. Several studies involving gonadal histomorphology are beginning to raise awareness for a more regular use of such approaches. Examples of such studies exist for sardines, *Sardinella brasiliensis* (Isaac-Nahum et al., 1988) and *Sardinops sagax* (Claramunt and Roa, 2001), for the Argentinean sand perch, *Pseudopercis semifasciata* (Macchi et al., 1995), for the tinfoil barb, *Puntius schwanenfeldii* (McAdam et al., 1999), for the tilapia, *O. niloticus* (Srisakultiew, 1993), for the ehu, *Etelis carbunculos*, and for the kalekale, *Pristipomoides sieboldii* (DeMartini, 2002).

General Aspects of Testis Histophysiology

Teleost testis shows greater morphological variation than in other vertebrates, as recognized for long (Dodd, 1972; Lofts and Bern, 1972; De Vlaming, 1974; Callard et al., 1978). In most cases, the testes are elongated-paired organs attached to the dorsal body wall. One main sperm duct (*vas deferens*) arises from the posterior mesodorsal surface of each elongated testis and, running between the rectum and the urinary ducts, ends in an opening at the urogenital papilla; which may be incorporated into the intromittent organs used by some species for copulation, as it is the case of the gonopodium found in viviparous teleosts.

Testicular microanatomy in fish is variable among species, although two basic types, lobular (Fig. 18.7) and anastomosing tubular, can be identified according to the differentiation of the germinal tissue (Grier, 1981); the former consisting of a collection of blind-ended sacs (lobules), and being the typical one for higher teleosts. Atherinomorph fishes have a special type of lobular testis (also called restricted spermatogonial testis type), correlated with several of their reproductive modifications such as sperm-bundle formation and internal fertilization. In spite of such differences, spermatogenesis occurs within roundish cysts formed (enclosed) by Sertoli cells (Table 18.2: A-D), and following from asynchronous to strict synchronous developmental patterns. It is widely assumed that true spermatogoonial stem cells are part of the testis, but even if definitive proofs are lacking contemporary data continues to endorse the ideia (Kuwahara et al., 2003; Chaves-Pozo et al., 2005). Initial cysts form by mitotic proliferation of spermatogonia, which originate spermatocytes that, undergoing the first meiotic division, give light to the secondary spermatocyes. These stages complete the second meiotic division and originate spermatids. Undergoing differentiation (spermiogenesis), the spermatids form the spermatozoa. The duration of

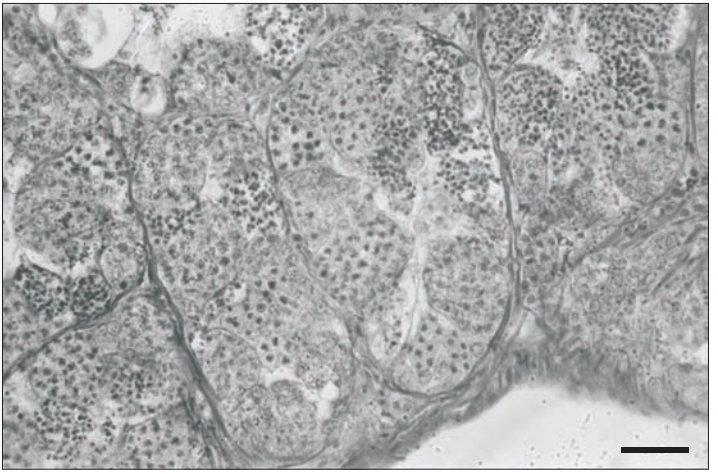
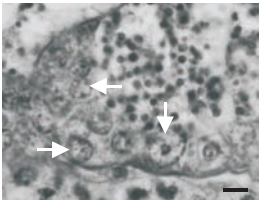
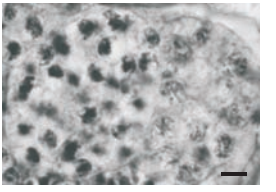
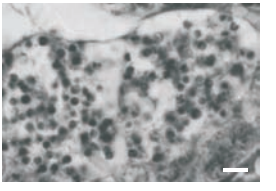
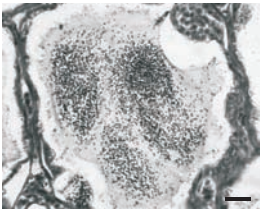
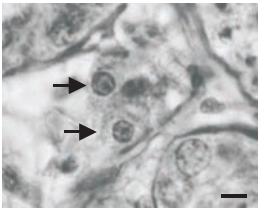


Fig. 18.7 Histological section of a testis of the lobular type, demonstrating a close relationship between Sertoli cells and germ cells, at various stages of development, forming round and easily recognizable spermatogenic cysts. Interstitial (Leydig) cells are located in the connective tissue (not seen at this magnification). Haematoxylin-eosin staining. Bar = 70 μm.

spermatogenesis is species specific (Nagahama, 1983). The mature sperm are released into a central lumen (Table 18.2: D), which ultimately leads them to the efferent ducts. Sperm are mixed in secretion fluid, which can be dense to bind the sperm together (Grier and Fishelson, 1995; Fishelson, 2003). There is a complement of somatic cells dedicated to the physical maintenance and functional regulation of spermatogenesis, including the cited Sertoli cells (also called sustentacular, intralobular, or cyst cells) and the Leydig (interstitial, or interlobular) cells.

The fish testis is typically gonochoristic and occasionally hermaphroditic. For instance, in agnathan the testicular tissue exists as a single cord, and in the particular case of hagfish (Myxinidae) it commonly contains ovarian tissue too (Gorbman, 1983). Within the sperm tubules, germ cells develop within follicles that finally break to release sperm into the body cavity, and from there to the exterior, via an original breach in the 'cloacal wall'. Sertoli cells are found in direct association with germ cells, which they support physically and nurture by modifying the chemical microenvironment. The ultrastructural features of the Sertoli cells suggest phagocytosis and an involvement in metabolite transport; their function is equivalent to that described for ovarian granulosa cells (Hoar and Nagahama, 1978) (Fig. 18.2). The relationship between Sertoli

Table 18.2 Overview of spermatogenesis in the tilapia, *O. mossambicus*. Haematoxylin-eosin staining.

Cell types	*Main cellular characteristics*	*Histological images*
Spermatogonia	These appear as pale cells located within the basal region of the spermatogenic cyst (spermatocysts). They are usually the largest germ cells within the testis. Scattered mitotic figures can be detected. Spermatogonia spermatocyts form. Bar = 7 μm.	A 
Spermatocytes	These cells can reach half the size of the spermatogonia, and their cytoplasm tends to be more basophilic. The nucleus of primary spermatocytes is large and the chromatin is irregurlarly condensed (at right). Cells enter in metaphase I (at left). Bar (B) = 7 μm.	B 
Spermatids	These cells become much smaller than the spermatocytes. The nucleus is the major component of these cells, and so they are strongly basophilic. Mitotic figures are obviously not found at this stage. Bar (C) = 7 μm.	C 
Spermatozoa	They are characteristically very small and highly basophilic. Typically, they have a flagellum and occupy the lumen of the tubules. Most teleost testes are of the lobular type, and so there are no special associations with Sertoli cells. Those occur in the tubular-type testes. Bar (D) = 28 μm.	D 
Leydig (interstitial) cells	Leydig cells are not always easily recongized in fish, as they can appear as small scattered cells. They are located in the connective tissue, near blood vessels and fibroblasts. In tilapia they are easy to be located, as they are have a round nucleus and fairly abundant cytoplasm (arrows). Bar (E) = 5 μm.	E 

and germ cells is more or less intimate, considering the different species. For instance, in species such as the killifishes, *Fundulus* spp., the connection amongst the germ and the Sertoli cells are less tight than that observed for the majority of teleosts (Petrino et al., 1989). Sertoli cells become hypertrophic during late spermiation, which appears to be related with the synthesis of the MIS under the pituitary gonadotropin (GtH-II) control.

The Leydig cells (Table 18.2: E) are interspersed in the connective tissue surrounding the lobules or tubules with Sertoli-germ cell units; their primary function is to produce steroids needed for gametogenesis and expression of secondary sex features. Abundance of Leydig cells varies much among fishes (Nagahama, 1983). The 3ß-hydroxy-Δ5-steroid dehydrogenase (3ß-HSD) is an enzyme involved in steroid hormone synthesis that was histochemically demonstrated in the Leydig cells of many teleosts (Nagahama, 1983). Leydig cells primary function is the androgen synthesis needed for spermatogenesis and for expression of secondary sex characteristics (Hoar and Nagahama, 1978; Redding and Patiño, 1993); their function is equivalent to that described for ovarian theca cells (Hoar and Nagahama, 1978).

The hagfish testis contains germ cells at all stages of maturity and, thus, spawning is expected to be repetitive. In lampreys (Petromyzontidae) which die after spawning, spermatogenesis is a single synchronous event. So, sperm production in some teleosts is a single synchronous event, whereas in others it is cyclic or even continuous. Nevertheless, it is possible to verify that the endocrine control of male reproduction is quite similar among species (Fig. 18.2). Testis growth and development coincide with increased plasma levels of 11-ketotestosterone (11-KT), and to a lesser extent, testosterone (T) (Scott and Sumpter, 1988; Borg, 1994; Norberg et al., 2001). Recent studies revealed the existence of androgen and progestogen receptors in fish testes (Todo et al., 2000; Ikeuchi et al., 2001, 2002); the latter works are particularly interesting as to advancing our understanding of the mechanisms underlying sex steroid signalling.

In elasmobranchs, the testes present a peculiar cell arrangement and cyclic spawning is typical. For instance, in the dogfish *S. acanthias* the testes are paired structures within which the germ cells and their supportive Sertoli cells divide and develop together in numerous, discrete, spherical units called spermatocysts or *ampullae* (Dodd, 1983). The site of hormone synthesis in the testis of elasmobranchs has been the subject of

controversy. Pudney and Callard (1984) reported the presence of cells in the interstitial tissue that are morphologically analogous to Leydig cells occurring in the testes of higher vertebrates. They preferred to describe those cells as Leydig-like. However, a significant role for Sertoli cells in steroid production was proposed (Pudney and Callard, 1984; Callard, 1992). In fact, there are data supporting the fact that Sertoli cells are the primary steroidogenic elements of dogfish testis (Cuevas et al., 1993). In the spotted ray, *Torpedo marmorata*, Sertoli cells (like Leydig cells) showed the characteristic ultrastructural aspect of steroidogenic cells (Marina et al., 2002). Moreover, and in immature males, when Leydig cells displayed a fibroblastic-like morphophenotype, Sertoli cells already had the typical structure of steroidogenic cells. Synchronous waves of spermatocysts develop from the so-called 'germinal border' or 'germinal zone' of the testes, and mature in a methodical progression towards the inner parts of the organ. Irrespective of the zonate appearance of the testis, the ripe spermatocysts open and release sperm into an adjacent system of efferent ducts that transport them to external copulating organs, for internal insemination of females.

In vivo and *in vitro* studies show that 11-KT is most effective as a direct stimulator for spermatogenesis (Fig. 18.3) (Miura et al., 1991a, b; Borg, 1994; Cavaco et al., 1998). On the other hand, T is most effective as a stimulator of both hypothalamic and pituitary activity (Goos et al., 1986; Amano et al., 1994), but leading anyway to further activation of the testis (Dufour et al., 1983; Schreibman et al., 1986; Xiong et al., 1993; Montero et al., 1995). We know now that 11-KT also activates Sertoli cells to stimulate the production of activin B. *In vitro*, this protein induced the proliferation of spermatogonia in the same manner as 11-KT (Ikeuchi et al., 2001). The current view is that gonadotropin stimulates the secretion of the fish androgen (11-KT) from Leydig cells, which, in turn, activates Sertoli cells to produce mediating factors (e.g., insulin-like growth factor-I and activin B) that stimulate premitotic spermatogonia, thus putting spermatogenesis in motion. As to 11-KT regulatory action, the cDNAs encoding two androgen receptors (ARα and ARβ) were described for the first time in the testes of Japanese eel, *Anguilla japonica,* and of Nile tilapia, *O. niloticus* (Ikeuchi et al., 2001). It was further revealed that although both AR mRNAs are present in the eel testis prior to sexual ripeness, only ARα transcripts increase during spermatogenesis. These observations imply that ARα and ARβ play different roles in spermatogenesis (Ikeuchi et al., 2001); a question still at stake. Still, in the Japanese eel, *A. japonica*, spermatogenesis was shown to be elicited when there is suppression of the

expression of a 'preventing substance', named eSRS21 (Miura et al., 2002).

During spermiation, Leydig cells continue their steroidogenic activity under gonadotropic stimulation, although the conversion of 17-P into androgens by the enzyme 17,20-lyase is reduced. The adjacent spermatozoa are said to further utilize the unconverted steroid to produce the progestagen 17α,20β-dihydroxy-4-pregnen-one (17,20β-P) (Ueda et al., 1983, 1985). This apparent shift in the steroidogenic pathway from the production of androgens to the formation of 17,20β-P is further facilitated by an intratesticular positive feedback mechanism, in which the activity of 17,20-lyase in the somatic cells is inhibited by the 17,20β-P, resulting in an increase of precursors for further formation of 17,20β-P (Yaron, 1995).

It is important to note that spermatozoa within the seminiferous tubules are immotile and may lack fertilization capacity. Fish spermatozoa acquire their motility (in parallel with key metabolic changes) when passing through the sperm duct, in a process that seems to occur in a relatively short time (Morisawa and Morisawa, 1986). Similar to the female mechanism, an increase in plasma GtH-II levels at the onset of the spawning season causes a shift from the steroidogenic production of androgens by the testes towards the production of MIS (Nagahama, 1994).

GtH-II and the MIS induce increases in milt volume by stimulating production of seminal plasma (Marshall et al., 1989; Pankhurst, 1994), and the MIS further stimulates motility of the stored spermatozoa, namely via an increase in the pH of that seminal plasma (Miura et al., 1995; Ohta et al., 1997). Studies in the masu salmon, *Oncorhynchus masou*, have shown that the motility of sperm is acquired by the effect of 17,20β-P (but not testosterone or 11-KT) on the sperm duct, increasing the pH from about 7.5 to 8.0-8.5 (Fig. 18.3). This was related with an increase in spermatic cAMP concentration, allowing the acquisition of motility (Miura et al., 1992). Although data from salmonids confirm the dependence of sperm flagellar movement on cAMP (Cosson et al., 1991; Inaba et al., 1998), there are alternative and additional explanations for the acquisition of motility. In this vein, MIS are thought to have a direct effect on Na^+/K^+-ATPase activity in the sperm duct (Marshall et al., 1989, 1993).

Concerning the annual changes in fish testis, its maturation degree has been classified in diverse ways (Brown-Peterson et al., 1988; Crim and Glebe, 1990; Murphy and Taylor, 1990; Ceballos-Vázquez and Elorduy-

Garay, 1998) for instance, using the following traditional sorting classes: developing; mature; ripe, gravid and running ripe; spent; regressed, resting and recovering. Nevertheless, this organization yields relatively little information on the details of the dynamics of testicular growth and spermatogenesis. Besides, there has been a great variability in the terminology of maturation classes among authors and the terms used are often vague and subjective. Brown-Peterson et al. (2002) suggested a new terminology, based, on the one hand, in easily distinguishable histological changes in the germinal epithelium and, on the other hand, in the stages of germ cells that are present. Such strategy holds the potential for introducing a more standardized set of characteristics that can be used to determine reproductive classes in a large number of teleosts. There are a priori several advantages of using this new (germinal epithelium) classification, particularly for species with an extended spawning season, such as the multiple-spawning fishes. In true, these were classified as 'ripe' during the entire reproductive season (Brown-Peterson et al., 1988; Cueller et al., 1996; Ceballos-Vázquez and Eldorduy-Garay, 1998). Using maturation classes defined by changes in the germinal epithelium to describe testicular maturation, as advanced by Brown-Peterson et al. (2002) actually gives a more accurate picture than when using the referred traditional terminology. The new classification advances five maturation classes: early maturation; mid maturation; late maturation; regression; and regressed.

General Concepts and Morphofunctional Aspects of Hermaphroditic Species

A fish species is hermaphroditic when a substantial proportion of the individuals among a population are able to produce gametes of both sexes, either simultaneously or sequentially, at some time during their life. Hermaphroditism may occur when the fundamental antagonism that normally exists between male and female hormonal influences during development and at maturity 'breaks down'. In this case, neither male nor female sexual tissue is developmentally preferred or, conversely, switched off, and both develop (Price, 1984). For instance, in the mangrove killifish, *Rivulus marmoratus*, most young individuals contain only ovarian tissue but, with aging, there is an increasing development of testicular tissue in the gonads and so they become hermaphrodites (Cole and Noakes, 1997). The potential for both male and female development exists in hermaphroditic fishes, and genetic influences on hormonal control in the

regulation of hermaphroditism have been discussed (Kallman, 1984; Price, 1984).

Hermaphroditism exists in three ways: (1) protogyny, in which some or all individuals function first as females and, later in life, exclusively as males; (2) protandry, in which the sex change is from male to female; and (3) simultaneous hermaphroditism, in which individuals function simultaneously as both male and female. In the next paragraphs, several examples will illustrate all the three models. Irrespective of the concept and models, it is important to realize that many histological studies during decades revealed that the gonads of either protandrous or protogynous hermaphrodites have some gametes of the opposite sex; this could be confirmed when analysing fully mature gonads (Zohar et al., 1978, 1984; Kadmon and Yaron, 1985; Pollock, 1985; Micale et al., 1987; Chang and Yueh, 1990; Chang et al., 1994; Guiguen et al., 1994; Micale and Perdichizzi, 1994; Nakamura et al., 1994; Tobin et al., 1997).

As other groupers, the honeycomb grouper, *E. merra*, which have lunar-synchronized spawning cycles, displays protogynous hermaphroditism and mainly inhabit the coastal waters from temperate to tropical latitudes (Lee et al., 2002). In this species, the ovaries show a pattern of development similar to that described for the group-synchronous gonochoristics (Lee et al., 2002). In fact, like the rabbitfishes, which are strict lunar synchronized spawners, a minor release of eggs may occur just before or after the major spawning lunar day (Hoque et al., 1999; Rahaman et al., 2000a; Lee et al., 2002).

Sequential hermaphroditism (protogyny and protandry) is reported in Sparidae (Atz, 1964; Micale and Perdichizzi, 1994; Bruslé-Sicard and Fourcault, 1997; Perrot et al., 2000). The seabream, *Pagellus acarne*, exhibits protandric hermaphroditism, wherein all individuals first mature as males. Then, they undergo testicular regression and the initially immature ovarian zone becomes functionally female (Le-Trong and Kompowski, 1972; Lamrini, 1986; Pajuelo and Lorenzo, 1994, 2000). On the contrary, in the protogynous red porgy, *Pagrus pagrus*, males originate from females, with the ovaries showing a pattern of development similar to that described for multiple spawner gonochoristic species (males still having ovarian tissue containing some follicles capable of estrogen production) (Kokokiris et al., 1999, 2000).

Both-ways sex change (or bi-directional sex change) occurs in several polygynous species of some fish families, such as the *Gobiidae* and *Pomacanthidae* (Kuwamura and Nakashima, 1998; Sakai et al., 2003).

Reversed sex change occurs even in fishes often demonstrating to be protogynous in nature. Indeed, it is confirmed that the largest dominant male changes sexual behaviour and gonads back to those of a female when it becomes subordinated again, after cohabitation with a larger male (Sunobe and Nakazono, 1993; Kuwamura et al., 2002). Among polygynous fishes, bi-directional sex change only comes about in monochromatic species that maintain haremic mating systems (Kuwamura et al., 2002). However, it is unclear whether males of sexual dichromatic protogynous fishes can completely transform back to females. Exceptional among haremic fishes, some angelfishes maintain conspicuous sexual dichromatism, e.g., *Centropyge interrupta* and *C. ferrugata* (Moyer and Nakazono, 1978; Moyer, 1990). As the size advantage model has predicted, protogynous sex change happens widely in the genus (Moyer, 1990). Therefore, sex change is socially controlled by a dominance relationship among harem members (Moyer and Nakazono, 1978; Sakai, 1997; Sakai et al., 2003).

Presently, the mangrove killifish, *Rivulus marmoratus*, is the only known self-fertilizing hermaphroditic vertebrate (Harrington, 1961; Warner, 1978; Sakakura and Noakes, 2000). This species is of interest not only for its unique reproductive biology but also because of the genetically identical individuals within each self-fertilizing lineage, sometimes referred to as clones (Harrington, 1967, 1971; Harrington and Kallman, 1968). In general, mangrove killifish individuals are hermaphrodites and produce both sperm and ova simultaneously. Nevertheless, secondary males develop from the hermaphrodites by the loss of ovarian tissue, and primary males develop directly to produce sperm throughout the rest of their lives (Harrington, 1971; Soto and Noakes, 1994).

So far, few studies have reported the role of sex steroids in natural sex differentiation and sex control of hermaphroditic fish (Lone et al., 2001). A study conducted in several hermaphrodite species revealed 11β-hydroxytestosterone (11β-HT) as the predominant steroid in the plasma, whereas 11-KT was not detected (Idler et al., 1976).

In the goldlined seabream, *Rhabdosargus sarba*, a protandrous hermaphrodite, the plasma pattern of sex steroids is similar in male and intersex individuals. However, the intersex individuals have higher levels of conjugated E_2, of conjugated androstenedione, and of free T, than do the females, even during the spawning period. This may indicate that to have a sex change from male to female, the estrogen levels have to be higher to suppress the antagonistic effects of androgens (Yeung et al.,

1993). Nevertheless, some species display the normal teleost pattern of steroid levels, with higher levels of serum 11-KT in males than in females (Nakamura et al., 1989, 1994; Cardwell and Liley, 1991a, b; Cochran and Grier, 1991; Kime et al., 1991; Godwin and Thomas, 1993).

Other protandrous hermaphrodite fish is the sobaity, *Sparidentex hasta*. Curiously, during its sexual maturation period, only the levels of both E_2 and 11-KT can be used to sex the fish, since those of T are definitely not particular of one gender (Kime et al., 1991). More recent studies in this species have revealed that their gonads differ from those of the other hermaphrodite species described so far (Lone et al., 2001). In fact, the gonad of a sexually mature sobaity, *S. hasta*, is homogeneous, both morphologically and histologically, in the sense that an individual, whether male or female, has only one kind of germinal tissue, i.e., either testicular or ovarian (Lone et al., 2001). This observation concurs with the first hormonal studies conducted in this species by Kime et al. (1991).

A Brief Note on Unisexual Species

Unisexual fishes are rare, but there are examples where females produce only female offspring, such as in *Poecilia* spp. and in *Poeciliopsis* spp. (Turner, 1982). This may occur in nature or in the laboratory through the processes of gynogenesis or hybridogenesis, whereby the reproduction of unisexual forms is accomplished with the participation of males from closely related bisexual species (Schultz, 1979). In gynogenesis, the entire chromosomal complement (2n) of the female is preserved while the genetic contribution from the male is eliminated. Hybridogenetic fishes exist as permanent hybrids of two closely related bisexual species, in which whereas gametic fusion occurs (and both maternal and paternal genomes are expressed) all the chromosomes of male origin are eliminated during oogenesis. Therefore, gametes of only maternal origin are produced, and the hybrid genome is restored when such females are crossed with males of the closely related species (Price, 1984). Of the two processes, gynogenesis is more common, although both mechanisms are rare in nature (Price, 1984).

Liver Function and Fish Reproduction—Key Aspects

When talking about breeding-related morphofunctional changes in fish, the liver, and especially the hepatocytes, immediately come to mind as they govern the production of both yolk precursors and eggshell components, namely the well-known vitellogenin and the zona radiata

proteins. A throughout review on both aspects was recently made by Arukwe and Goksøyr (2003). Another important aspect is the liver role in steroid metabolism. Thus, hepatocytes do have their own histophysiology of reproduction, reflecting their workload to coupe with the needs. Synchronous and group-synchronous species (especially salmonids) are much better studied then other groups, and especially when compared with asynchronous species. On revising the issue of seasonal changes in hepatocytes and their correlation with the endocrine system (Rocha et al., 2003), it suffices to say that current advanced research greatly focus on molecular mechanisms regulating expression of estrogen and aryl hydrocarbon receptors, cytochrome P450 1A, vitellogenin and eggshell proteins, and how they are being disturbed by pollutants that mimic endogenous steroids (Arukwe et al., 2002; Boon et al., 2002; Bemanian et al., 2004).

Anyway, a few selected considerations will be made, essentially to recall relevant events and metabolic pathways that are most relevant, and that would deserve more interdisciplinary study, namely comparing fishes with the different breeding strategies outlined along the text of this Chapter.

Hepatocytes can govern the plasmatic levels of quite a different number of molecules. Most compounds absorbed by the intestine pass through the liver, via the portal venous system, being eventually captured by the hepatocytes and then processed, originating other metabolites. By other hand, as in the higher vertebrates, various hormones modulate the later mechanisms in fish. For instance, E_2, which can greatly change with the breeding cycle, does influence hepatic carbohydrate metabolism. This was studied (though not too intensively) in several species, and is mainly expressed as a fall of glycogen content (per g liver) and increased glycolysis with rising levels of E_2 and parallel gonadal maturation (Petersen and Emmersen, 1977; Olivereau and Olivereau, 1979; Leatherland, 1985; Busby et al., 2002).

In the course of the breeding cycle, lipid deposition occurs in somatic tissues. Self-control of fattening has been largely studied under the perspective of endocrine regulation of either circadian or circannual cycles of metabolism. Therefore, when fuels are abundant, fatty acids derived from the diet or synthesised by the liver are esterified, and secreted into the blood as very low-density lipoproteins. These are the main source of fatty acids used by adipose tissue to synthesise the triacylglycerols, and in fishes the all dynamics of the process has a vital

importance in reproduction, manly during the vitellogenic period. However, studies on the effects of E_2 in lipogenic enzymes have yielded inconsistent results. In addition, substantial differences concerning the hepatocyte content in lipids were not consistently highlighted among species with synchronous, group-synchronous and asynchronous ovarian development (Hori et al., 1979; Peterson et al., 1983; Truscott et al., 1986).

In very recent studies, and when studying a multiple-batch group-synchronous, the Nile tilapia, *O. nilotica*, and the group-synchronous brown trout, *S. trutta fario*, we noticed several interspecific differences in the relationship between the pattern of hepatocyte morphology versus ovary and testes development (still unpublished observations). New data will soon be brought to light on such liver morphofunctional differences between breeding patterns, namely as to hepatocyte size and number variances, as well as to hepatocytic organeollar content and handling of energy reserves. Anyway, our current work allow us to tell that there are numerous fundamental questions to be explored in liver, as to structure versus function under diverse breeding patterns. A virtually unexplored field is definitely the fish liver local neural regulation and cell functional cross-talk, via paracrine action. This latter aspect gains relevance in view of the advances in mammals, showing that both paracrine and autocrine signalling are critical for the cooperation of liver cells in health and disease (Kmiec, 2001; Marzioni et al., 2005).

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CHAPTER

19

Current Perspectives on 17β-Estradiol (E2) Action and Nuclear Estrogen Receptors (ER) in Teleost Fish

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ABSTRACT

Estrogen action in both female and male fish has been well documented as having multiple reproductive and non-reproductive roles. In this chapter, we shall outline the numerous effects of estrogens in teleosts, underscoring differences between fish and mammals. Interestingly, aromatase activity in the brain is reported to be much higher in fish than in other taxa, thus, a model for localized neuroestrogen synthesis is presented. The principal mediators of estrogen action are the estrogen receptors (ERs) and genome duplication during the evolution of teleost fish have led to multiple estrogen receptors in cyprinid and salmonid fish, perhaps providing functional diversity in estrogen receptor mediated responses. We will also review what is currently known

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about estrogen receptor ontogeny and regulation of expression in adult tissues. We explore the application of novel and powerful molecular techniques, for example, microarrays and reporter gene assays, to study the effects of estrogens at a molecular level. Both *in vitro* and *in vivo* binding assays have provided detailed insight into interactions between endogenous and exogenous ligands and the estrogen receptor isofoms. These techniques are now being applied to questions in the emerging field of toxicogenomics and issues that have been at the forefront of research in endocrine disruption. We summarize some of the mechanisms of estrogenic endocrine disruption and suggest important areas of future investigation that have been largely overlooed in the literature to date.

Key Words: 17-β estradiol; Teleost; Receptor evolution; Reporter gene; Environmental estrogens.

OVERVIEW

17β-Estradiol (E2) plays key roles in neural development, growth, sexual maturation, and control of reproductive processes in both female and male vertebrates, in addition to regulating cellular proliferation in normal and cancerous tissues. Teleost fish are useful models for the study of E2 action because they exhibit diverse and alternative reproductive strategies, from gonochorism to hermaphroditism and unisexuality (Blázquez et al., 1998a,b). Another attractive aspect of teleost models concerns the direct neural control of pituitary function. In contrast to mammals, teleosts do not have a functional portal blood system. A multitude of neurotransmitters and neuropeptides directly innervate the pars distalis of the anterior pituitary and stimulate or inhibit gonadotropic hormone release. This direct innervation is considered to be a derived rather than primitive condition since elasmobranchs have a primitive version of a mammalian-like hypothalamo-hypophysial portal system (Gorbman, 1995). During the course of teleost evolution, it has been suggested that the median eminence migrated into the anterior pituitary, resulting in direct innervation of the anterior pituitary (Peter et al., 1990). This anatomical feature has proven to be useful in determining the origin of hypophysial systems controlling reproduction, as well as elucidating the mechanisms of both positive and negative feedback action of estrogens and other sex steroids.

Also of interest is that many teleosts are polyploids, including members of the salmonid and cyprinid families, because of a series of genome duplication events occurring approximately 550 and 450 million

years ago (Ohno, 1970; Meyer and Schartl, 1999). This was most likely followed by additional gene duplications in many fish species (Aparicio, 2000). Thus, teleost fish offer a unique opportunity to study the gain of novel protein functions through relaxed selection pressure of a duplicated gene. Some possible examples are the presence of distinct ovarian and brain cytochrome P450 aromatases (CYP19) (Tchoudakova et al., 1998, 2001) and multiple estrogen receptors (ERs; Hawkins et al., 2000; Menuet et al., 2002) in fish.

The principal mediator of E2 action is the estrogen receptor (ER), a nuclear transcription factor (NR3A). The first estrogen receptor, the E2 receptor alpha (ERa or NR3A1) was cloned from human (Green et al., 1986; Greene et al., 1986) and chicken (Krust et al., 1986). The first ER in fish was cloned and sequenced shortly thereafter (Pakdel et al., 1989). It was thought that only one estrogen receptor existed in vertebrates until a second form, the E2 receptor beta (ERβ or NR3A2), was cloned from rat prostate (Kuiper et al., 1996). The two E2 receptors show amino acid sequence similarity of >90% in the DNA binding domain and approximately 60% in the ligand-binding domain across fish species (Kelley and Thackray, 1999; Tchoudakova et al., 1999; Ma et al., 2000). The ERα/β subtypes are thought to have arisen from a gene-duplication event prior to the diversification of the ray-finned fishes more than 400 million years ago (Kelley and Thackray, 1999; Thornton, 2001). More recently, a novel third ER has been identified in Atlantic croaker (ERγ) (*Micropogonias undulatus*) (Hawkins et al., 2000) and zebrafish (ERβ2) (*Danio rerio*) (Bardet et al., 2002; Lassiter et al., 2002).

We briefly review the neuroendocrine control of reproduction in fish, as well as the synthesis and functions of E2, in order to provide the background and physiological context for a more detailed consideration of the structure, function, evolution, tissue distribution, and regulation of nuclear ERs in teleost fish. It should be noted that E2 action in mammals is also potentially mediated through membrane-bound receptors but these are not yet characterized in fish. We also discuss the consequences of exposure to estrogenic endocrine disrupting chemicals (EDCs) on sexual development and reproduction. While EDCs with estrogenic or antiestrogenic activities in the environment have received a considerable amount of attention, a new and emerging concern is that of pharmaceuticals and personal care products in the environment. For example, 17α-ethinylestradiol (EE2), an active ingredient of many birth

control pills, has been detected both in water (Ternes et al., 1999; Kolpin et al., 2002) and sediments (Holthaus et al., 2002) of aquatic ecosystems at levels that elicit E2–dependent biological effects in fish (Larsson et al., 1999). Lastly, we outline two powerful molecular approaches, reporter gene assays and cDNA arrays, currently used to evaluate the risk of estrogenic exposure to fish populations.

HYPOTHALAMO-PITUITARY AXIS

The neural control of pituitary function in fish has been reviewed extensively (Kah et al., 1993; Trudeau, 1997; Trudeau et al., 2000) and will only be covered briefly here. The decapeptide gonadotropin-releasing hormone (GnRH) is the primary hypothalamic neurohormone that stimulates the release of gonadotropins from the anterior pituitary. Multiple GnRH forms arise from distinct genes in teleosts (Parhar et al., 1996) and stimulate the release of gonadotropins via different signal transduction pathways (Chang et al., 2000). Both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) homologues exist in teleosts and are also referred to as gonadotropin-I and gonadotropin-II, respectively (Querat, 1994). Gonadotropins stimulate gametogenesis and the synthesis of gonadal sex steroids, such as testosterone (T) and E2 which, in turn, feed back on the hypothalamo-pituitary axis to regulate sexual maturation and spawning. The principal inhibitor of LH release is the catecholamine dopamine. More than 20 other neurohormones have been shown to regulate LH release in fish (Trudeau, 1997) and include a multitude of neuropeptides, classical neurotransmitters and growth factors, some of which are targets for estrogen action in the brain.

SYNTHESIS OF ESTRADIOL

Ovarian and Testicular Synthesis

The first enzymatic step in steroidogenesis is catalyzed by cytochrome P450scc (side chain cleavage), which is located on the inner mitochondrial membrane. Cytochrome P450scc converts cholesterol, the precursor of steroid hormones, to pregnenolone. Subsequent processing of pregnenolone by members of the mono-oxygenase family and hydroxyl-steroid dehydrogenases occurs in the smooth endoplasmic reticulum and culminates in the production of various androgens, including testosterone (T). T itself may be converted to E2 by another cytochrome P450 19α

(CYP 19a) through aromatization of the cholesterol A-ring. In female teleosts, E2 production in the ovary involves a two-cell process: first, gonadotropins, acting through a cAMP-dependant mechanism, stimulate the production of T in ovarian thecal cells. T is then transported to ovarian granulosa cells where it is aromatized to E2, and subsequently released into the blood (Nagahama, 1983). Circulating plasma E2 typically ranges between 2-4 ng/ml in non-reproductive teleosts and are elevated to 5-20 ng/ml during pre-spawning periods (Scott et al., 1983; Sineros et al., 2004). Onuma et al. (2003) have recently showed that circulating E2 plasma levels in pre-spawning chum salmon (*Onchorhychus keta*) can vary dramatically from year to year, ranging between 15 ng/ml to 35 ng/ml.

In salmonid ovarian follicles and testes, two distinct membrane gonadotropin receptors have been identified (Yan et al., 1992; Miwa et al., 1994). In ovary, the Type I gonadotropin receptor binds FSH and LH, and is expressed in both thecal and granulosa cells during vitellogenesis (oocyte growth) when FSH levels are higher and E2 is being synthesized. However, Type I gonadotropin receptor is expressed only in thecal cells and connective tissue during oocyte maturation when LH levels are higher and maturation-inducing hormone (MIH; 17α,20β-dihydroxy-4-pregnen-3-one) is being synthesized (Nagahama, 1997). The Type-II gonadotropin receptor binds only LH and is exclusively expressed in granulosa cells during oocyte maturation. Thus, in salmonids, it appears that both FSH and LH have the potential to stimulate E2 synthesis through the gonadotropin Type-I receptor during vitellogenesis.

Ovarian synthesis of E2 declines towards the end of vitellogenesis and throughout oocyte maturation, concurrently with an increase in synthesis of MIH (Planas et al., 2000), a steroid which is critical for germinal vesicle breakdown and that prepares the oocyte for successful fertilization (Nagahama, 1997). There is now convincing evidence to suggest that the increase in MIH in salmonid ovarian follicles is stimulated by the interaction between LH and the Type-II gonadotropin receptor found in granulosa cells (Planas et al., 2000). This receptor-ligand interaction results in the upregulation of 20β-hydroxysteroid dehydrogenase, the enzyme that catalyzes the final step of MIH synthesis (Nagahama, 1997). Interestingly, no role for FSH or LH in the decrease of aromatase activity has been demonstrated, and it has yet to be determined as to which factors lead to the overall decline in E2 synthesis by ovarian follicles (Planas et al., 2000). However, a decrease in both P450-aromatase mRNA levels

and, therefore, E2 synthesis occurs as oocyte maturation progresses (Nagahama, 1997).

Androgens, in addition to serving as a precursor to ovarian E2, regulate E2 production. Androgens have been shown to inhibit E2 synthesis by a suppressive action at multiple sites in the steroidogenic pathway in several vertebrate species. Braun and Thomas (2003) showed that androgens suppress testicular E2 production by a rapid, cell surface receptor-mediated mechanism in the Atlantic croaker. These results indicate that both genomic and non-genomic mechanisms are involved in the reciprocal inhibitory control of T and E2 in the fish testes.

There is also strong evidence that metabolic and stress hormones affect ovarian steroidogenesis, both directly in the ovarian follicle and indirectly by influencing pituitary gonadotropin synthesis and release. Thyroid hormones act synergistically with gonadotropins to stimulate ovarian development in several fish species (Cyr and Eales, 1996). Physiological levels of triiodothyronine (T3) directly enhanced gonadotropin-induced ovarian E2 synthesis in rainbow trout (Cyr and Eales, 1988) and Japanese medaka (*Oryzias latipes*) (Soyano et al., 1993), suggesting that T3 may be involved in regulating E2 synthesis in fish. Growth hormone (GH) also stimulates ovarian E2 production by potentiating LH action (reviewed by Le Gac et al., 1993; Trudeau, 1997). Furthermore, insulin-like growth factor-I appears to have differential effects on gonadotropin-induced steroidogenesis in thecal and granulosa cells in coho salmon (*Oncorhynchus kisutch*; Maestro et al., 1997) and common carp (*Cyprinus carpio*; Behl and Pandey, 1999). In rainbow trout, stress-induced cortisol production indirectly inhibits ovarian E2 synthesis, perhaps by decreasing LH in the pituitary (Teitsma *et al.*, 1998).

The main steroidogenic cell in the teleost testis is the Leydig cell, as in mammals (Fostier et al., 1983). Gonadotropin receptors are located on both Leydig and Sertoli cells in fish (Schulz et al., 2001). Miwa et al. (1994) have shown that the Type-I gonadotropin receptor will interact with both FSH and LH while the Type-II interacts specifically with LH. Moreover, throughout testicular development in salmon, Type-I receptors are localized to Sertoli cells. In contrast, Type-II receptors are found mostly in Leydig cells of spermiating fish only (Miwa et al., 1994). Aromatase mRNA is expressed in testis, generally at lower levels when compared to ovaries of females, in several fish species including channel catfish (*Ictalurus punctatus*; Trant et al., 1997), tilapia (*Oreochromis*

niloticus; D'Cotta et al., 2001), rainbow trout (*Oncorhynchus mykiss*; Govoroun et al., 2001b), and sea bass (*Dicentrarchus labrax*; Blázquez and Piferrer, 2004), although an immunohistochemical study in rainbow trout using specific antibodies to rainbow trout aromatase failed to detect the enzyme in any testis cell-types (Kobayashi et al., 1998). It has been proposed that repression of aromatase expression and, therefore, E2 synthesis, by various mechanisms (including temperature and androgens) is required for differentiation of testis during gonad development in fish (D'Cotta et al., 2001; Govoroun et al., 2001a).

Neuroestrogen Synthesis

The vertebrate brain is a site of estrogen production and teleost fish have exaggerated brain cytochrome P450 aromatase (CYP19β) activity compared to mammals. For example, neural aromatase activity in the goldfish (*Carassius auratus*) is 100-1000 times that of rodent and human brains (Callard et al., 2001). In goldfish, the activity of aromatase is correspondingly high in pituitary, but in the ovary it is 10% of that of the brain. The functional significance of high neuroestrogen synthesis remains unknown, however, Callard et al. (2001) suggested that the remarkable level of neuroestrogen synthesis in teleost brain is related to the regenerative capacity and neuroplasticity of the adult fish central nervous system (CNS). A neuroprotective role of aromatase and E2 has been documented in mammals (Azcoitia et al., 2001). Moreover, peak aromatase activity occurs around the organizational period of brain development and may be implicated in the development of sexually dimorphic structure and function in neuroendocrine regions in mammals and fish (Lephart, 1996; Menuet et al., 2003).

In teleosts, ovarian aromatase (CYP19α) and brain aromatase (CYP19β) are encoded by separate genes and are differentially expressed and regulated. In particular, the goldfish CYP19β promoter has two estrogen-response elements (ERE), whereas CYP19α does not. This is consistent with the observation that E2 treatments upregulate aromatase activity in the brain but not in the ovary (Gelinas et al., 1998; Kishida and Callard, 2001; Tchoudakova et al., 2001).

Of considerable significance to the regulation of neuroestrogen synthesis and action is the relative distribution of ERs and CYP19β in the brain. Forlano et al. (2001) were the first to demonstrate that brain aromatase was localized predominantly if not exclusively to radial glial

cells in the plainfin midshipman (*Porichthys notatus*) brain. Teleost neurons do not appear to express aromatase, which is in marked contrast to mammals and birds. Glial localization of aromatase was later confirmed in rainbow trout by Menuet et al. (2003). Gonzales and Piferrer (2002, 2003) have characterized brain aromatase activity and seasonal variations in the brains of European sea bass. *In situ* and immunocytochemical studies in the rainbow trout brain revealed a striking lack of co-localization of the ERα and CYP19β signals (Menuet et al., 2003). For example, high levels of both ERα and CYP19β were found in the preoptic area and hypothalamus but ERα was found in neurons and CYP19β in radial glial cells in the ependymal layer. Additionally, ERβ could not be detected in ependymal cells in zebrafish brain using *in situ* hybridization (Menuet et al., 2002). It is currently difficult to exclude the possibility that glia may express low levels of functional ERs, nevertheless, it is likely that E2 acts indirectly or via another ER subtype to alter glial aromatase in the fish brain. Moreover, this model of neuroestrogen action would suggest that T is locally converted to E2 in brain regions with high glial aromatase activity. Estrogen diffuses out of glia to modulate neuronal function by interacting with ERα or ERβ in neighboring target neurons. E2 upregulates CYP19β expression and many aspects of neuronal function in the preoptic area and hypothalamus. Moreover, autoregulation of ER production by E2 in fish and mammals is well documented (Le Drean et al., 1995, Agarwal et al., 2000; Rune et al., 2002). The possibility of a multilevel feedback mechanism of neuroestrogen synthesis and action should be investigated (Fig. 19.1).

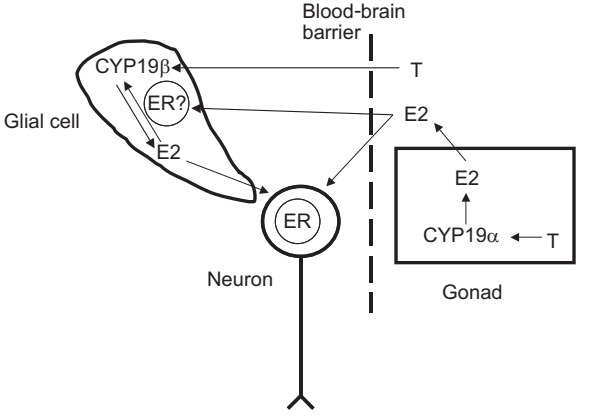

Fig 19.1 A proposed model for neuroestrogen synthesis and action in the brain.

ESTRADIOL HAS REPRODUCTIVE AND NON-REPRODUCTIVE ROLES

E2 Regulation of Gametogenesis

Oocyte growth involves the uptake of the yolk protein precursor vitellogenin (VTG) from the plasma into the oocyte (van den Hurk and Peute, 1979). During oocyte growth, a layered and highly differentiated eggshell or egg envelope is formed (Nagahama et al., 1994). The major component layer of the egg envelope is the zona radiata (ZR), which comprises three proteins designated α-, β-, and γ-ZR proteins (Hyllner et al., 1991; Celius and Walther, 1998). E2 acts directly on the hepatocytes to stimulate VTG synthesis (Ng and Idler, 1983; Takemura and Kim, 2001). This is a direct response to E2 since the presence of a functional ERE in the VTG promoter is well established (Teo et al., 1999). E2 also stimulates ZR gene expression in parallel with VTG and ER expression, suggesting that autoregulation of the ER is involved in a positive feedback effect of E2 to stimulate oocyte growth (Oppen-Bernsten et al., 1992; Knudsen et al., 1998; Arukwe et al., 2002). VTG is a calcium binding protein and calcium is required for VTG synthesis by rainbow trout hepatocytes (Yeo and Mugiya, 1997). In Atlantic salmon (*Salmo salar*) (Persson et al., 1998) and nibbler fish (*Girella punctata*) (Suzuki et al. 2000), E2 may act on scales or bone to mobilize calcium stores by stimulating osteoclast activity to increase calcium availability levels during vitellogenesis. ERs are present in goldfish scales, suggesting that E2 acts directly to regulate calcium mobilization in this tissue (Suzuki et al., 2000; Suzuki and Hattori, 2002). In contrast, in the marine gilthead seabream (*Sparus aurata*), E2 increases calcium plasma levels by increasing uptake from the environment through the intestine and gills with no effect on scale calcium content (Guerreiro et al., 2002).

Historically, E2 has been considered a 'female' hormone (Hess et al., 1997). This classification is under considerable scrutiny since it has been reported that concentrations of E2 in the fluids of the rete testis of bovine and rodent species can reach levels as high 250 pg/mL (Hess et al., 1997 and ref. therein) and in male teleosts, plasma E2 can be in the low ng/ml range (Onuma et al., 2003; Sisneros et al., 2004). Further support for the importance of E2 in spermatogenesis has been provided by the findings that male ERα and aromatase knock out rodents have impaired fertility, a consequence of defective efferent duct and germ cell development, respectively (O'Donnell et al., 2001). To date, the majority of our

knowledge about the potential roles for E2 in spermatogenesis has been derived from information gained from work carried out in mammalian species. More recently, however, ERα and/or ERβ have been identified in the testes of several teleost species including seabream (Socorro et al., 2000), channel catfish (Wu et al., 2001), medaka (*Oryzias latipes*) (Kawahara et al., 2000) and eelpout (*Zoarces viviparus*) (Andreassen et al., 2003).

The expression of ER in the testes of many different vertebrate species has long implicated a role for E2 in spermatogenesis (Wu et al., 2001 and refs. therein). For example, it has been reported that E2, through ER, promotes spermatogonial stem cell renewal in the testes of the Japanese eel (*Anguilla japonica*) (Miura et al., 1999). E2 can also suppress both basal GnRH-stimulated testicular T and 11-ketotestosterone (11-KT) production in goldfish (Trudeau et al., 1993c), perhaps by affecting the expression of steroidogenic enzymes, as has been shown in the male rainbow trout (Govoroun et al., 2001b). Interestingly, E2 itself has been found to induce ER levels in a cell specific manner in eelpout testes (Andreassen et al., 2003), a finding that may explain the adverse effects of environmental estrogens on testicular development and subsequent function (Jobling et al., 1996; Christiansen et al., 1998). In addition, Loomis and Thomas (2002) showed that *in vitro* incubation of Atlantic croaker testes with E2 coupled to bovine serum albumin resulted in rapid decreases in gonadotropin-induced 11-KT production, suggesting a non-genomic action of E2 in fish testes mediated by a membrane ER. Thus, in addition to its more 'traditional' role in oocyte development, there is mounting evidence from teleost studies to suggest a physiological role of E2 in testicular function.

Feedback to the Hypothalamo-pituitary-gonadal Axis

E2 exerts both positive and negative feedback on the brain and pituitary, tightly controlling LH production and release (Kah et al., 1997; Trudeau, 1997; Blázquez et al., 1998a; Melamed et al., 1998) and FSH release (Vetillard et al., 2003). In both male and female adult goldfish, E2 potentiates GnRH and taurine-stimulated LH release (Trudeau, 1997). In rainbow trout, ERs are present in the hypothalamus and pituitary and E2 treatment upregulates ER expression in hypothalamus but not in pituitary (Kah et al., 1997). This suggests that an E2-autoregulatory mechanism is involved in steroid feedback at the level of the hypothalamus (Kah et al.,

1997). Furthermore, the Chinook salmon (*Oncorhynchus tschawytscha*) LHβ subunit gene contains EREs and LHβ expression increases in response to E2 in a dose-dependent manner (Liu et al., 1995). Negative feedback actions of E2 on LH release in goldfish, rainbow trout and African catfish (*Clarius gariepinus*) involves activation of inhibitory dopamine neurons (Trudeau et al., 1993b; Linard et al., 1995; Blázquez et al., 1998a). Vetillard et al. (2003) determined that in gonadectomized female rainbow trout, E2 replacement stimulated the expression of tyrosine hydroxylase, the rate limiting enzyme in dopamine synthesis. The authors suggested that E2 upregulation of tyrosine hydroxylase may be a mechanism for negative feedback on LH and FSH production. E2 will also mediate the reproductive process via modulatory feedback on amino acid neurotransmitters in the brain. For example, in some teleost fishes, γ-aminobutyric acid (GABA) has an indirect stimulatory role in LH release and acts to stimulate GnRH release (Trudeau et al., 1993a; Trudeau, 1997; Mañanos et al., 1999). GABA is produced from the excitatory neurotransmitter glutamate by the enzyme glutamic acid decarboxylase (GAD). In the goldfish, E2 modulates brain GAD mRNA expression (Bosma et al., 2001) and in rainbow trout, the distribution of GAD expressing neurons strongly overlap with ERα-expressing cells in the preoptic region and the mediobasal hypothalamus (Anglade et al., 1999). These studies suggest that GABAergic neurons are a major target for E2 feedback on LH release.

E2 Affects Thyroid, Growth, and other Physiological Axes

Reproduction and thyroid function are interrelated in teleosts, as indicated by fluctuations in blood hormone levels over the reproductive cycle, and effects of hormone administration (Cyr and Eales, 1996). In salmonids, it has been demonstrated that E2 administration results in overall depression of thyroid function (thyroidal status). Most notably, E2 depresses plasma thyroid hormone levels in salmonids by affecting deiodination activity in liver and other tissues (Cyr and Eales, 1996). For example, in rainbow trout, E2 decreased plasma T3 levels by substantially reducing hepatic deiodination of thyroxine to the active T3 (Cyr et al., 1988). It has been hypothesized that the effect of E2 to decrease the thyroidal status is a reflection of resource partitioning, allowing only one energetically expensive process such as reproduction (associated with E2) or somatic growth (associated with thyroid hormones) to occur at a given

time during the annual cycle (Cyr and Eales, 1996). E2 biphasically modulates pineal melatonin secretion (Begay et al., 1994), suggesting that there are other mechanism by which E2 regulates seasonal hormone release.

E2 treatment increases circulating GH levels in goldfish and rainbow trout (Trudeau et al., 1992; Holloway et al., 1997; Zou et al., 1997) at least in part by increasing pituitary responsiveness to GH-releasing factors such as GnRH and thyrotropin-releasing hormone (Trudeau et al., 1992). Moreover, increased GH content (Zou et al., 1997) and forebrain mRNA levels of GH-releasing factors such as neuropeptide-Y (Peng et al., 1994) are also likely to contribute to increased blood GH levels following E2 treatments. In addition to increasing pituitary responsiveness to GH-releasing factors, E2 could also decrease pituitary responsiveness to inhibitors of GH release, such as somatostatin. For example, in rainbow trout, E2 treatment prevents *in vitro* somatostatin-induced inhibition of pituitary GH release (Holloway et al., 1997). E2 treatment also decreases circulating somatostatin-14 and somatostain-25 levels (Holloway et al., 1997, 2000) and decreases hypothalamic preprosomatostatin (PSS)-1 and PSS-2 mRNA levels in the rainbow trout (Holloway et al., 2000), indicating another route by which E2 may affect GH-release. In goldfish, however, although E2 treatment increases serum GH levels, it increases PSS-1 and PSS-3 mRNA levels but did not affect PSS-2 mRNA levels in forebrain (Canosa et al., 2002) and increases levels of mRNA encoding somatostatin-1 and somatostatin-5 receptors in pituitary (Canosa et al., 2003), effects which would be expected to decrease GH release rather than increase it. It is difficult to compare the results on PSS-1 and PSS-2 because Holloway et al. (2000) studied trout hypothalamus and Canosa et al. (2002) examined goldfish forebrain expression. Nevertheless, there are likely tissue- and species differences in the effects of E2 on somatostatinergic system. A possible example of the effect of increased GH following E2 treatments is the stimulation of cartilage growth in tilapia (*Oreochromis mossambicus*) (Ng et al., 2001).

Other non-reproductive roles of E2 include salt balance and regulation of the immune response. E2 treatment prevented freshwater-adapted tilapia from recovering plasma osmolality after transfer to 50% seawater (Vijayan et al., 2001). Activities of several liver and gill enzymes were depressed, including gill Na+/K+-ATPase. E2 and other sex steroids also have effects on the immune system in fish. For example, in common carp, E2 injection decreased phagocytosis by head kidney macrophages, as

well as their ability to produce nitric oxide and superoxide anion, an action which mimics that of cortisol (Watanuki et al., 2002).

To summarize, E2 plays a central role in female reproduction in its control of oocyte growth by its stimulation of synthesis of proteins incorporated into the developing egg and by feedback to the hypothalamo-pituitary axis to control its own production. In males, E2 affects spermatogenesis in the testes and affects the expression of proteins involved in the production of sperm and sex steroids. Apart from the hypothalamo-pituitary axis, E2 exerts additional actions which, nonetheless support reproductive processes, from calcium homeostasis to energy partitioning.

ERS ARE NUCLEAR TRANSCRIPTION FACTORS

ER Structure and Mechanism of Action

The ERs are nuclear transcription factors that form homodimers before binding to a DNA motif called an estrogen-response element (ERE). The ERs have distinct domains that include an NH_2- terminus (referred to as the A/B domain), a DNA-binding domain (DBD) (C domain), a hinge region (D domain), and a ligand-binding domain (LBD) (E/F domain) (Fig. 19.2). The NH_2- terminus contains a sequence known as activation function-1 (AF-1) which is involved in ligand-independent activation of the receptor. Interestingly, the first fish ERα identified appeared to be a truncated receptor that showed constitutive activity in the absence of a ligand. However, a full-length teleost ERα variant was later identified that contains a domain that has the function of repressing AF-1 activity (Pakdel et al., 2000). This is in contrast to mammalian ERs. Mezaki et al. (2001) demonstrated that medaka ERα, when activated in a ligand-independent manner, has a higher activity than human ERα when expressed in HeLa cells, suggesting that fish ERs are more active in the absence of a ligand. The DBD region of the receptor binds the ERE (a two zinc-finger motif in the gene promoter region) and activates gene transcription. The consensus ERE sequence derived from multiple estrogen-responsive promoters is 5'-GGTCANNNTGACC-3' (Sanchez et al., 2002). The LBD region contains the activation function-2 (AF-2) which operates in a ligand-dependent manner.

Studies in mammalian systems have shown that gene transcription is facilitated by proteins called co-activators that interact with the AF-1 and

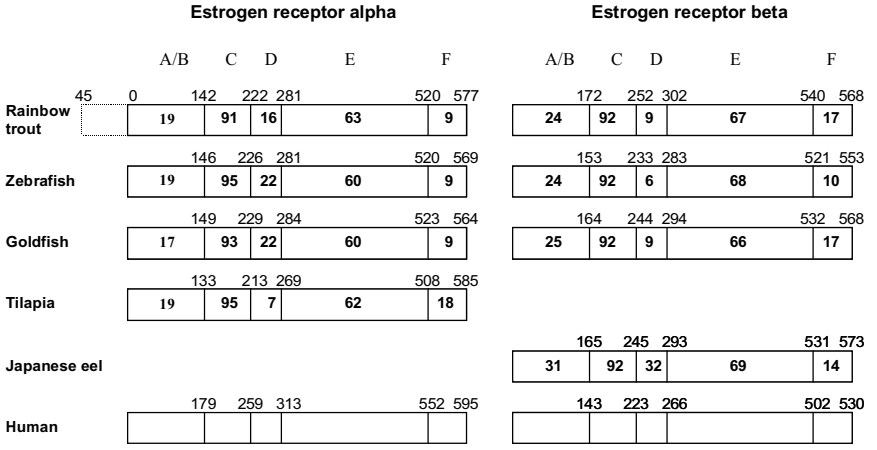

Fig. 19.2 Schematic comparison of teleost and human estrogen receptor isoforms (percent similarity to human ER in bold). Teleosts represented are members from the salmonid, cyprinid, percifid, and anguillid families. The A/B domain is the N-terminus, the C domain is the DNA binding domain, the D domain is the variable hinge region, the E domain is the highly conserved ligand binding domain, and the F domain is the C-terminus. Also shown are the corresponding position of the amino acids of each domain. The dashed lines (rainbow trout alpha receptor) represent the extended A/B domain that is found in most tissues.

the AF-2 domains to relax chromatin structure primarily through histone deacetylation. Conversely, co-repressors reduce the rate of transcriptional processes by stabilizing chromatin structure (Govind and Thampan, 2001). In trout, additional proteins have been shown to interact with the ERs to promote gene transcription. For example, the orphan receptors, COUP-TF1 and ARP-1, interact with the rainbow trout ERα in the presence of E2 to enhance autoinduction of the ER gene (Lazennec et al., 1997). This is in contrast to previous reports in mammals which indicated that these proteins predominantly inhibit transcriptional processes. Furthermore, Metivier et al. (2000) demonstrated that this cooperative interaction between ERα and COUP-TF1 is variable in the presence of different xenoestrogens. For example, interactions between the trout ERα and COUP-TF1 in the presence of the estrogenic compound 4-nonylphenol were significantly reduced when compared to E2. The trout glucocorticoid receptor (GR), a mediator of the stress response, has also been shown to interact with trout ERα and inhibits E2–stimulated transcription of an ERE-luciferase construct in an *in vitro* cell transfection system, which demonstrates crosstalk between the reproductive and stress

axis at the molecular level (Lethimonier et al., 2002). Further studies involving ER-mediated transcription in additional species of fish will undoubtedly lead to the discovery of novel protein interactions and will elucidate the effects of estrogens on diverse molecular processes.

Ligand-binding Characteristics of the ERs

Tissue sensitivity to E2 is determined at least partially by ER number and affinity (K_d). Receptor numbers in tissues are influenced by many factors such as age (Carreau et al., 1984), sexual stage (Pottinger, 1988), and the levels of endogenous or exogenously administered hormones (Lazier et al., 1985; Mann et al., 1987). A number of different studies have shown that the affinity of the rainbow trout hepatic nuclear/cytosolic ER for E2 generally ranges from K_d values of 2 to 13 nM (Campbell et al., 1994; MacKay et al., 1996; Tremblay et al., 1998; Tollefsen et al., 2002). Other teleost ERs also exhibit K_d values within the same range as that reported for rainbow trout: 2 to 6 nM in Atlantic salmon (hepatic, nuclear/cytosolic; Lazier et al., 1985); 1.4 to 2.1 nM in common carp (hepatic cytosolic; Kloas et al., 2000); 0.9 to 2.4 nM in spotted seatrout (*Cynoscion nebulosus*) (hepatic, nuclear/cytosolic; Smith and Thomas, 1989).

The aforementioned studies tested receptors isolated from teleost ovarian and hepatic tissues, and although Scatchard analyses showed one population of receptors, it is possible that different ER subtypes are present in these preparations. Studies of single-recombinant channel catfish ER subtypes indicate that the affinity of ERβ for E2 (K_d of 0.2 nM; Xia et al., 2000) was approximately one order of magnitude higher than ERa (liver; Xia et al., 1999), suggesting that ERβ may be able to respond to relatively low levels of estrogen, while ERα may require higher ligand concentrations to be activated (Xia et al., 2000). Zebrafish ERs produced *in vitro* from expression vectors containing the entire ER coding region showed that the ERβ2 (see section below) has a 1.8-fold higher affinity for E2 than ERα and ERβ1 (Menuet et al., 2002). However, Atlantic croaker ER produced from expression vectors containing most of the ER coding regions for ERβ and ERγ showed similar affinities for E2 (1.4 nM and 1.2 nM, respectively). It is likely that there are potential differential binding characteristics of the ER subtypes within and among teleost species. However, it should be noted that this may be dependent upon the technique used to evaluate ER-binding affinities to E2. Future studies should include the development of standardized methods to measure ER binding affinities.

ER Evolution

Molecular phylogenetic analysis has suggested that the ER is the most ancient of the nuclear steroid receptors and is likely the progenitor of the other steroid receptors (Baker, 2001). Diversification of a duplicated ER coupled to a ligand exploitation process likely led to multiple nuclear transcription factors in the steroid receptor superfamily (Thornton, 2001). Recently, an estrogen-responsive protein has been identified in *Aplysia californica,* the first reported for an invertebrate, suggesting that the E2 signaling system dates back more than 600 million years (Thornton et al., 2003). Since the first teleost estrogen receptor was identified in rainbow trout (Pakdel et al., 1989), ERs from Japanese eel (Todo et al., 1996), channel catfish (Xia et al., 1999), gilthead seabream (Munoz-Cueto et al., 1999; Socorro et al., 2000), rainbow trout (Menuet et al., 2001), goldfish (Tchoudakova et al., 1999; Ma et al., 2000), and Atlantic salmon (Rogers et al., 2000) have been cloned and characterized. In addition to the presence of these two subtypes in teleosts, a third receptor, ERγ, has been identified in Atlantic croaker (Hawkins et al., 2000) and zebrafish (Bardet et al., 2002; Lassiter et al., 2002). We used the NCBI database to blast Atlantic croaker ER nucleotide sequences against the pufferfish (*Fugu rubripes*) genome and found three putative ER sequences, an ERα (SINFRUP00000062437) and two additional sequences similar to Atlantic croaker ERβ (SINFRUP00000067205) and ERγ (SINFRUP00000071164), suggesting the presence of three functional ERs in pufferfish. Molecular phylogenetic evidence suggests that two serial duplications of an ancestral steroid receptor occurred before the divergence of the lamprey (*Petromyzon marinus*) and jawed vertebrates, which most likely gave rise to the ERα and ERβ existing in vertebrates (Thornton, 2001). Moreover, phylogenetic trees that include ERγ show that this receptor has a higher sequence homology to ERβ than to ERα and is most likely derived from an ancient ERβ receptor (Menuet et al., 2002). As a result, the ERγ has also been designated ERβ2 (Bardet et al., 2002) and ERβa (Lassiter et al., 2002) by some authors. However, it is unclear whether ERγ is derived from a gene-specific duplication of ERβ or a genome duplication event (Hawkins et al., 2000).

ER Ontogeny and Tissue Expression

A significant role of E2 is to mediate developmental processes via the estrogen receptors. Bardet et al. (2002) detected zygotic expression of all

three ERs between 48 and 72 hours post-fertilization in zebrafish. Developing embryos contain maternal steroids that promote the initiation of gonadal development and sex differentiation. In particular, T is present at high levels in the embryo and diminishes after the emergence of the gonads (Hines et al., 1999). In contrast, levels of E2 remain low until after ovarian development, suggesting that aromatization of T is critical for gonadal development and sex differentiation. In the zebrafish CNS, the expression of CYP19β and the onset of estrogen responsiveness occurs at a similar developmental time interval, suggesting that both ligand and receptor are endogenously produced at the end of segmentation and beginning of hatching when morphogenesis and cellular differentiation is occurring (Callard et al., 2001). Temperature will affect the mRNA expression of the ERα and ERβ, as well as CYP19β (Tsai et al., 2003). During tilapia development, expression of CYP19β increased before posthatch day 10 at high temperatures but decreased, along with the expression of the ERα, at lower temperatures. Between posthatch days 10 and 20, only ERβ was influenced by temperature while at posthatch day 30, only aromatase was affected by higher temperatures, suggesting that the temperature sensitivity of these genes is stage-dependent. The influence of temperature on sex differentiation and brain development is well documented in both reptiles and fish species (Rhen and Lang, 1994; Blázquez et al., 1998a,b). Thus, it is likely that differential expression of the ERs in response to environmental signals is an important factor contributing to gonadal and brain development.

Estrogen receptors are prominent in diverse tissues throughout many developmental and reproductive stages in teleosts (Table 19.1). Atlantic salmon parr express ERα in many tissues, including brain and liver (Rogers et al., 2000), and juvenile catfish express both ERα and ERβ in the testis, ovary, spleen, liver, intestine, gill, skin, and blood (Xia et al., 2000). However, it is presently unclear whether there exist additional ER isoforms that are expressed during development in these species. In adult teleosts, there is wide overlap between the ER subtypes and both ERα and ERβ are expressed in the brain, liver, spleen, gills, interrenal, and skin. A noted exception is that ERβ appears to be less pronounced in brain tissue of goldfish (Tchoudakova et al., 1999) and Atlantic croaker (Hawkins et al., 2000). However, in the zebrafish brain, the ER subtypes are abundant in many brain regions and exhibit both overlapping and localized distributions (Menuet et al., 2002). Pakdel and colleagues (2000) report

Table 19.1 Some examples of ER isoform tissue distributions in different species of teleosts. See text for more details on nomenclature for cloned ERβ and ERγ in teleost fishes. NR = not reported

	testes	*ovary*	*liver*	*brain*	*pituitary*	*intestine*	*gill*	*interrenal*	*spleen*	*skin*	*blood*	*muscle*	*heart*
[1]*Heteroptneustes fossilis*	α β	α β	α β	NR	NR	α β	α β	α β	α β	NR	α β	NR	NR
[2]*Salmo salar*	α β	α β	α β	α β	NR	NR	NR	NR	NR	NR	NR	NR	NR
[3]*Micropogonius undulatus*	α β γ	α β γ	α β	α β γ	NR	NR	NR	NR	NR	NR	NR	α γ	NR
[4]*Sparus aurata*	α β	α β	α β	α	NR	β	NR	β	NR	β	NR	NR	α β
[5,6]*Carassius auratus*	α β1	α β1	α β 1+2	α β 1+2	α β 1+2	β	NR	NR	NR	NR	NR	α β 1+2	α β 1+2
[7]*Oncorhychus mykiss*	α	α	α	α	α	NR	NR	NR	NR	NR	NR	NR	NR
[8]*Danio rerio*	α β 1+2	α β 1+2	α β 1+2	α β 1+2	α β 1+2	α β 1+2	NR	NR	NR	NR	NR	NR	NR

(1) Xia et al. (2000) (2) Rogers et al. (2000) (3) Hawkins et al. (2000) (4) Socorro et al. (2000) (5) Tchoudakova et al. (1999) (6) Ma et al. (2000) (7) Menuet et al. (2001) (8) Menuet et al. (2002)

on a truncated N-terminal ERα variant (ERαs) in rainbow trout that was detected only in the liver, suggesting that this variant of ERα may be exclusively involved in vitellogenin production, or other liver-specific functions. The recent isolation of the two isoforms $ER\beta_1$ and $ER\beta_2$ in addition to ERα in goldfish, adds an additional dimension for studying tissue-specific regulation of gene expression by E2 (Ma et al., 2000). Studies in goldfish found that males and females had the highest expression of ERα in the pituitary, with significantly lower levels in the brain, ovary, testis, liver, muscle, heart, and intestine (Choi et al., 2003). In contrast, goldfish $ER\beta_1$ was found at higher levels in the ovary, testis and liver with lower levels in the forebrain, mid/hindbrain, pituitary, retina, muscle, and heart (Tchoudakova et al., 1999; Choi et al., 2003). $ER\beta_2$ was predominantly expressed in the pituitary, telencephalon, hypothalamus, and liver of female goldfish (Ma et al., 2000). The recently discovered ERγ has a more restricted tissue distribution, showing high expression in ovarian/testicular tissue and low expression in the brain and liver of Atlantic croaker (Hawkins et al., 2000). It should be pointed out that many of the aforementioned studies on ER tissue distribution are based on RT-PCR and caution should be taken when comparing ER levels across tissues and species. Future studies should continue to address tissue expression patterns throughout development and reproductive cycles using additional techniques (real time PCR, quantitative in situ hybridization) before general trends can be drawn.

Fish offer a unique opportunity to study the estrogenic effects in tissues not present in terrestrial organisms. For example, the gills have been shown to express both ERα and ERβ (Xia et al., 2000). Using transgenic medaka embryos, Kawamura et al. (2002) demonstrated that overexpression of ERα and exposure to estrogenic chemicals resulted in disruptions in blood clotting and yolk vein formation, suggesting that the highly vascularized gills may be sensitive to estrogens. Disruption of development in the transgenic fish was rescued by treatment with the ER antagonist tamoxifen, indicating a receptor mediated effect. Transgenic medaka embryos developed normally when estrogen exposure occurred after early neurula stages, indicating that the sensitive stage is before neurulation. The complexity of temporal and spatial expression patterns of the multiple ER subtypes and isoforms poses a significant challenge for elucidating the roles of ERs in physiological processes, such as development and reproduction.

ENDOCRINE DISRUPTION BY ESTROGENIC CHEMICALS

Estrogenic Pollutants in the Environment are a Threat to Fish

Many synthetic compounds are present in the aquatic environment at levels that alter the action, production, and metabolism of E2. Of particular concern are compounds that pass into freshwater systems as a result of industrial waste, urban sewage and agricultural runoff. A recent publication by the International Programme on Chemical Safety (2002), an expert group assembled on behalf of the World Health Organization, the International Labour Organization and the United Nations Environment Program, reviewed the global, peer-reviewed scientific literature regarding environmental exposure and adverse outcomes via mechanisms of endocrine disruption. It was concluded that '*endocrine disruption is undoubtedly occurring in wild fish populations in North America, Asia, Australia, and Europe and is caused by a variety of mechanisms including hormone receptor interactions, interference with the biosynthesis of sex steroids and perturbations of the hormonal control by the pituitary on reproductive and adrenal processes*'. It has become clear that the health risks associated with anthropogenic sources of pollution is also an issue at the forefront of fish conservation and biology.

The impact of estrogenic EDCs on the molecular and cellular processes involved in fish reproduction has been well documented in both laboratory and field-based experiments (Arcand-Hoy and Benson, 1998; Tyler et al., 1998; Arukwe, 2001). Disruption of the endocrine system can occur at either the organizational or activational stage of the lifecycle of an organism (Guillette et al., 1995). A contaminant that modifies the morphology or function of a tissue as the result of exposure during a particularly sensitive period of development is said to have an organizational effect. For example, administering EDCs before or during early sex differentiation has been demonstrated to alter phenotypic sex (Blázquez et al., 1998b; Madigou et al., 2001; Afonso et al., 2002). EDC exposure during this labile period may be particularly detrimental to the breeding dynamics of fish populations. In contrast, if an EDC transiently alters the function of mature tissue, it has induced an activational effect. A well-studied example is the production of VTG in male fish upon exposure to environmental estrogens (Sumpter and Jobling, 1995; Gronen et al., 1999). The following sections will briefly review the known

mechanisms of environmental estrogen action and discuss potential factors responsible for the wide variety of biological effects observed in fish exposed to estrogenic EDCs. Finally, we review some current methods used for assessing estrogenic EDC exposure in fish.

The Effects of Estrogenic EDCs vary with Development, Sex, and Species

Sexual development and reproduction are characterized by marked changes in hormone concentrations, receptor activity and overall cellular responses to endocrine stimuli. Therefore, the type and degree of modulation induced by an EDC may depend on the stage of lifecycle at which exposure occurs. The organizational effects of EDCs are of particular concern since they can be induced with low concentrations, are usually irreversible, and can result in abnormal formation and function of affected tissues and organs. Rasmussen *et al.* (2002) demonstrated that an EDC exposure can affect the embryonic development *in ovario* in the viviparous eelpout *(Zoarces viviparus)*. Early mRNA expression of estrogen–sensitive genes can also be influenced by xenoestrogens. Post-hatch exposure in teleost fish has been shown to modulate genes such as ERα and brain CYP19β (Tsai et al., 2001). Various studies have attempted to elucidate the critical period of developmental sensitivity to xenoestrogen exposure by measuring such parameters as gene induction, sex ratios, gonad morphology, and reproductive behaviour (Blázquez et al., 1998a; Krisfalusi and Nagler, 2000; Iguchi et al., 2002). Early xenoestrogen exposure is also thought to enhance the sensitivity of these animals to a repeated exposure as adults and affect reproductive behaviour through ER-mediated mechanisms (Nimrod and Benson, 1997; Foran et al., 2002). Transgenerational studies have also been important in determining the long-term reproductive potential in the offspring of exposed fish (Foran et al., 2002; Schwaiger et al., 2002).

In the effort to predict the toxic effects of estrogen mimics in different vertebrate species, it is generally assumed that chemicals interact with the nuclear sex hormone receptors in a similar way regardless of the species in question. Using a radioreceptor binding assay, Tollefsen et al. (2002) determined that various estrogenic chemicals (e.g., pharmaceuticals, pesticides, and industrial chemicals) interact with the ER of Atlantic salmon and rainbow trout with similar affinity and specificity. However, work by Wells and Van Der Kraak (2000) demonstrates that the existence

of a substantial interspecies difference in the binding of endogenous steroids and chemicals to the androgen receptor, both between species (rainbow trout and goldfish) and even between tissues of the same species. Furthermore, evidence from studies using ER fusion proteins from different species suggests that the rainbow trout ER differs significantly from the mammalian and amphibian ERs in the ligand-binding requirements of xenoestrogens (Matthews et al., 2000). The presence of multiple ER isoforms in target tissues and between species, and the identification of novel mechanisms of xenoestrogen action proves a difficult hurdle to overcome when designing endocrine screening assays to be used across affected species.

Few studies address the effect of gender on the response of an organism to estrogen exposure in fish. Bosma et al. (2001) demonstrated that both E2 and T significantly increased mRNA expression of the GABA synthesizing enzyme GAD65 in the female goldfish hypothalamus while decreasing expression in the male hypothalamus. Moreover, differences in sensitivity to the toxic effects of E2 and octylphenol, a xenoestrogen, was demonstrated in immature male and female goldfish (Blázquez et al., 1998a). E2 and octylphenol both induced 50% mortality among male goldfish at concentrations that produced no mortality in female goldfish. Such experiments demonstrate that there are major developmental and sex differences in responses to both endogenous hormones and EDCs. This underscores the necessity to determine sex-specific effects in future research.

Screening Estrogenic EDCs with Reporter Gene Assays

ER reporter gene systems are based on a ligand binding to an endogenous ER or transfected ER which subsequently initiates the transcription of a reporter gene whose activity can be quantified (Zacharewski et al., 1995). Human breast cancer cell lines (e.g., MCF-7, T47D or ZR-75) with substantial levels of endogenous ERs have been successfully used in sensitive and highly responsive reporter gene assays to screen the estrogenic activity of environmental compounds (White et al., 1994; Martin et al., 1995; Legler et al., 1999). There are limited reporter gene assays using fish ERs transfected into mammalian, yeast or fish cell lines to detect environmental estrogens. However, the majority of these studies in fish only test ERα activity, and studies testing ERβ, ERγ and other isoforms of ER subtypes are few.

Transcription assays with ERE-luciferase based reporter gene systems have revealed species differences to various xenoestrogens. Legler et al. (2002) reported that zebrafish ERβ and ERγ showed higher transactivation by xenoestrogens relative to E2 than human ERβ in a human embryonic kidney (293HEK) cell line. In contrast, zebrafish ERs have been shown to be less sensitive to some selective estrogen receptor modulators than homologous mouse ERs transfected into rat osteosarcoma (ROS17/2.8) cells (Bardet et al., 2002). It should be noted that the discrepancy between species may partially reflect the inherent differences in activities of the host cell lines employed. For example, fish cells may contain unique ER co-activators (i.e., COUP-TF1) for ER-dependent processes and efforts should be placed on developing all-fish cell reporter systems, so that the roles of the multiple piscine ERs in mediating E2 and xenoestrogen actions can be elucidated.

Examples of the major ER reporter gene assays employed to detect environmental ER ligands are presented in Table 19.2. For example, the transient transfection assay by Ackermann et al. (2002) employs an expression vector containing the complete rainbow trout ERα cDNA and an ERE-regulated reporter gene construct in a rainbow trout gonad cell line (RTG-2). This bioassay has a wide detection range (0.05 nM to 5 nM E2) and has the advantage of being a fish-specific bioassay. A chimeric receptor/reporter gene assay has also been established for both mammalian and non-mammalian vertebrates (Zacharewski, 1997; Matthews et al., 2002). This bioassay involves transiently transfecting two vectors into human breast cancer cell lines. The first vector contains the DNA-binding domain of the Gal4 yeast transcription factor linked to the rainbow trout ER ligand binding domain, resulting in the constitutive expression of a chimeric ER. The second vector (17m5-G-Luc) contains the Gal4 regulated reporter construct linked to the firefly luciferase cDNA reporter gene, which is only activated by the ligand-bound chimeric receptor. This bioassay has been shown to be less sensitive to serum-borne estrogens and provides researchers with greater selectivity for examining binding of environmental estrogens to the ER ligand binding domain. In summary, differences in responsiveness between bioassays with different receptor isoforms and the use of mammalian cell lines rather than fish cell lines should be a concern when examining the fish-specific effects of environmental estrogens.

Table 19.2 Examples of reporter gene assays developed for fish estrogen receptors

Assay System	*Receptor Construct*	*Reporter Gene Construct*	*Transfection Reagent/ Cell Line*	*Reference*
ERE-regulated reporter genes	complete rainbow trout ER	ERE-Tk-Luc	Superfect (branched polycationic); rainbow trout gonad (RTG-2)	Ackermann et al. (2002)
	complete rainbow trout ER	ERE-Tk-Luc	calcium phosphate; chinese hamster ovary (CHO-K_1)	Lethimonier et al. (2000)
	complete rainbow trout ER	PGL3-TATA-5xERE-Luc	Lipofectamine (liposome formulation), human cervical cancer (HeLa)	Sumida et al. (2003)
Chimeric receptor-based				
	Gal4 yeast transcription factor fused upstream of DEF domains of rainbow trout ER	17m5-G-Luc	calcium phosphate; human breast cancer (MCF-7)	Zacharewski et al. (1995)
Yeast-based				
	complete rainbow trout ER	1-3xERE-URA3-lacZ	lithium acetate; yeast (BJ-ECZ)	Petit et al. (1995)
	complete rainbow trout ER	FP3-EREp-CYC1-lacZ	lithium acetate; yeast (BJ2168)	Madigou et al. (2001)

Expression Profiling of Environmental Estrogen Action

High throughput analysis of gene expression using DNA array methods is becoming a powerful diagnostic tool to evaluate the transcriptome response to environmental estrogens. Briefly, an array contains an alignment of genes (targets) on a fixed surface (e.g., nylon membranes, glass or plastic slides) that hybridize to samples (probes) containing complementary DNA labeled with a fluorescent dye or radioactive ^{32}P. The microarray format is particularly useful because expression patterns of thousands of genes are determined simultaneously, thus producing a molecular 'fingerprint'. Novel genes and pathways responding to environmental estrogens can be discovered and characterized.

Using microarrays as a diagnostic tool to characterize the molecular response to E2 and estrogenic compounds is not new for the study of mammalian systems (Choi et al., 2001; Naciff et al., 2002). Although large-scale gene microarrays (>1000s of genes) for fish are now available (Ton et al., 2003; Gracey et al., 2004), studies investigating gene responsiveness to E2 and xenoestrogens have only been done on a smaller scale. Larkin et al. (2002) produced an array containing 132 putative E2-responsive genes isolated from the liver of largemouth bass by differential display PCR. The authors determined gene expression profiles for E2 and two estrogenic pollutants, nonylphenol and p,p'DDE in largemouth bass (*Micropterus salmoides*). Although similar trends in induction of known estrogen-responsive genes (e.g., choriogenin types 2 and 3 and VTG types 1 and 2) were observed between the three ligands, there were differences in the level of induction. For example, both VTG1 and VTG2 were increased 100-300, 30-100 and 2-3 fold in males exposed to E2, nonylphenol, and p,p' DDE, respectively. The differences observed were most likely due to variation in ligand binding affinity and the doses of the three compounds administered to the fish. However, many unknown expressed sequence tags were induced by E2 but suppressed by the pollutants, demonstrating that nonylphenol and p,p'DDE are not purely estrogenic and may have other effects. It should be noted that a concern with interpreting gene expression data involves the dose of the estrogenic compound. Chronic exposure to low doses of an estrogenic compound may result in more dramatic changes in gene expression and may be more detrimental to the individual than a single acute dose (Denslow et al., 2001).

Difficulties with the large amounts of gene expression data collected include the identification of patterns of gene expression following

exposure to estrogenic chemicals. Furthermore, gene expression data does not always correlate with protein levels, and although gene expression profiling is a powerful tool used to monitor and identify estrogen responsive genes, it must be integrated with information on post-transcriptional processes. Therefore, genomic and proteomic approaches for specific fish tissues should be considered. In the future, the development of tissue-specific arrays in teleost fish will reveal novel actions and mechanisms of estrogens and estrogenic chemicals. Our research has begun to address this and we have developed a brain derived CDNA array to study E2 action (www.auratus.ca)

Conclusions

Teleost fish represent the largest vertebrate group with approximately 25,000 species worldwide. Teleosts have diverse reproductive strategies and have provided insight into many conserved and novel functions of E2. In both males and females, E2 exerts strong positive feedback effects on the hypothalamo-pituitary axis to enhance LH release from the pars distalis of the pituitary. Interestingly, the teleost brain has a remarkable capacity to produce neuroestrogen and, although the role of E2 in feedback regulation is reasonably well documented, the role of neuroestrogen in the vertebrate brain remains to be fully understood. It can be hypothesized that locally produced E2 in the CNS participates in the regulation of the neuroendocrine system controlling LH release. However, there are likely other roles, for example in neuronal growth, plasticity, protection, and repair, in addition to effects on neurotransmission mediated through a membrane receptor for E2. Teleost models provide insight into ligand-nuclear receptor interactions and the co-repressors modulating transcriptional activation of E2-responsive genes. In particular, the concept of ER autoregulation is well understood in the trout model. Teleost tissues express many novel variants and isoforms of the three ER subtypes. The relative roles and potential interactions of ERα, ERβ and ERγ remain to be determined. Lastly, it may be expected that fish contain membrane-bound receptors important for mediated E2 action and this will undoubtedly be addressed in future studies.

Many fish species are sensitive to the endocrine disrupting effects of xenoestrogens. Indeed, the concept of endocrine disruption is largely based on the results of intense study of the effects of these chemicals on estrogen-dependent processes in teleosts. *In vivo* and *in vitro* bioassays

using fish or fish cells are now well recognized as being some of the most sensitive and useful in the study of environmental estrogens. Another molecular tool, expression profiling, is only in its infancy and will address the effects of E2 and estrogenic EDCs on the transcriptome. However, the expanding availability of teleost cDNA sequences and expressed sequence tags (genes whose function is not yet determined) from a number of developmental stages and tissues will undoubtedly contribute to our knowledge of reproductive and non-reproductive roles of E2.

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CHAPTER

20

Stress Biomarkers and Reproduction in Fish

Giulia Guerriero* and Gaetano Ciarcia

ABSTRACT

Fish exposed to stressful conditions both in the wild and in culture may differ in the extent of their physiological responses and reproductive consequences to stressors. A wide array of biomarkers used to demonstrate exposure and effects of stressors, has been briefly reviewed, especially in relation to reproduction. Furthermore, we stress the importance of antioxidants as stress biomarkers and propose to assay vitamin E, the major antioxidant in reproduction, during the different reproductive phases in sea bass.

Key Words: Stress; Fish reproduction; Biomarker; Antioxidants; Vitamin E.

INTRODUCTION

The word *stress* has been defined in several ways (Pickering, 1981). In the physiological approach, it is used as the response of the body, i.e., a

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physiological cascade of events, that occurs when the organism is attempting to resist death or re-establish homeostatic norms in the face of an insult (the actions of intrinsic or extrinsic stimuli, commonly defined as stressors).

Since the elements of the physiological stress response have been described elsewhere (Donaldson, 1981; Colombo et al., 1990; Barton and Iwama, 1991; Wendelaar Bonga, 1997), only a brief overview will be presented here, recognizing that much of our understanding of how fish respond to stressors is based on the study of juvenile life history stages. Extreme changes in the physical environment (temperature, turbidity, salinity), animal interaction (predation, parasitism, intensive competition for space, food or sexual patterns), human interference including aquaculture practices (netting, handling, transport and crowding) and water pollution (low water pH, heavy metals and xenobiotics) or reproduction constitute stress factors or stressors (Jones, 1995 reviewed in Guerriero et al., 2002, 2003). The physiological stress response begins following the perception of a stressful event. The actions of stressors produce effects that threaten or disturb the homeostatic equilibrium and/or elicit a coordinated set of behavioral and physiological responses aimed to be compensatory and/or adaptative, thus enabling the animal to overcome the threat (Chrousos and Gold, 1992). Physiological responses to a stressor are either specific, for a single stressor or a group of related stressors, or non specific, when they are commonly observed in reaction to many different types of stressors (Guerriero et al., 2003).

These responses typically involve all levels of animal organization and are known as 'integrated stress response' (Cannon, reviewed in Wendelaar Bonga, 1997). For the integrated stress responses in fishes, the distinction between primary, secondary and tertiary responses has been introduced by Pickering and Pottinger (1995). Primary responses consist in the activation of brain centers, resulting in the massive release of catecholamines and corticosteroids, whereas secondary responses are usually defined as the manifold immediate actions and effects of these hormones at the blood and tissue levels, including increases in cardiac output, oxygen uptake, mobilization of substrates and disturbance of hydro-mineral balance. Tertiary responses extend to the level of the organism and population: inhibition of growth, reproduction and immune responses, and reduced capacity to tolerate subsequent and additional stressors. The rigidity of this classification is difficult to reconcile with the

more recent evidence on the flexibility and complexity of stress responses in fish.

Stress response severity may vary, depending on the species to which the stress is applied. However, if an animal is experiencing intense and continue chronic stress, the stress response may lose its adaptive value and become dysfunctional, resulting in reduced resistance to pathogens, inhibition of growth but, especially, in reproductive failure (Mc Ewen, 1998; Schreck, 2000). Changes in tissue and organ functions that attempt to cope with or compensate for the stressor may differ among individuals in rate or magnitude, but share general characteristics in their mode and action starting from the general Adaptation Syndrome (Selye, 1950, 1973). As in mammals, fish responses can be quite polymorphic within and among species (Barton and Iwama, 1991). However, it can be also polymorphic with regard to the stage of maturity, gender, season, physical condition, social status, water quality and type of stressor (for review see Pankhurst and Van der Kraak, 1997; Wenderlaar Bonga, 1997; Schreck, 2000).

A great deal of research is going on in this field and substantial progress, with respect to the control and physiological role of the stress, can be expected in the next few years, using stress biomarkers.

BIOCHEMICAL EFFECTS OF STRESS AND RELATED BIOMARKERS IN FISH

The stress response in the individual fish involves adjustments at all levels of organization (molecular, biochemical, physiological, structural and behavioral) and these will result in effects at the population and ecosystem levels (Bartell, 1990). Traditionally, changes in growth, mortality rate and reproductive success have been used as indicators of environmental stress (Fig. 20.1). Monitoring with biological indicators (Fig. 20.2) can be used to help make inexpensive predictions regarding the chance (exposure), mechanism of action (effect) and uncertainty of response (susceptibility) to stressors and their significance in risk assessment has been recently reviewed by Schlenk (1999). However, this approach suffered from the major problem that irreversible damage to the fish population(s) may have occurred before any remedial action. Consequently, attention has been given to some biochemical changes in stressed fish, due to either effects mediated by stress hormones or specific responses to particular types of stress, because they may provide a valuable and sensitive early indication of environmental problems (Lam and Gray, 2003).

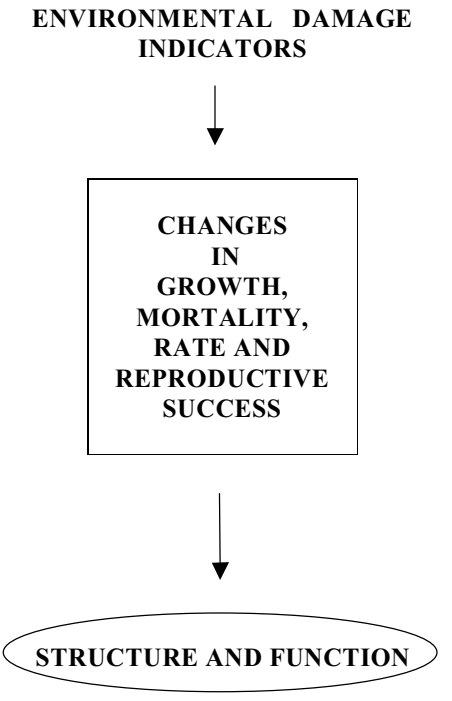

Fig. 20.1 Bioindicators of environmental stress.

When fish are subjected to stress, immediate neuroendocrine changes, known as primary stress responses, are dominated by changes in the sympatho-chromaffin system and the hypothalamo-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997).

Catecholamines are released both as neurotransmitters in the sympathetic nervous system and as classical hormones from the adrenal medulla or chromaffin tissue in fish. Their release under stress conditions are often associated with either respiratory strain, such hypoxia (Aota et al., 1990) or exhaustive exercise (Primmett et al., 1986), or social stress (Pottinger and Pickering, 1992; Pottinger et al., 1992).

The hypothalamo-pituitary-interrenal (HPI) axis in fish consists of a hierarchy of hormonal pathways including hypothalamic corticotropin-releasing factor (CFR) → pituitary adrenocorticotropin (ACTH) → interrenal corticosteroids with a series of regulatory feedback loops operating at different levels (Barton and Iwama, 1981, 1991; Donaldson, 1981; Roche and Bogè, 1996). The principal corticosteroid produced by

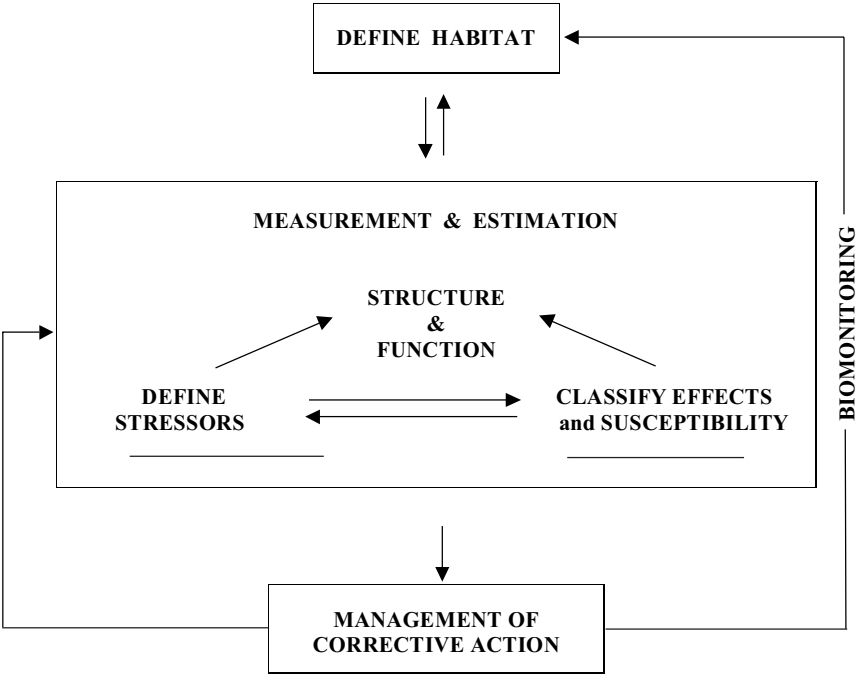

Fig. 20.2 A schematic role of biomonitoring.

fish is cortisol, which is synthesized and secreted in response to the many forms of environmental stress. In general, the kinetics of elevation, plateau and return to the baseline of plasma cortisol is somewhat slower than that of catecholamines and related to the different fish conditions (wild, domesticated, or hatchery-reared fish) (Fevolden et al., 1991; McGeer et al., 1991; Salonius and Iwama, 1991; Pottinger et al., 1992). Prolonged elevation can also have severe and debilitating consequences for disease resistance, growth and reproduction.

The current state of knowledge regarding stress in fish has been reviewed (Engel et al., 1999) and, although some information on the role of hormones is available, data on the effects of stress course are lacking. Thyroidal activity is generally suppressed by chronic forms of stress (Byamungu et al., 1990); circulating pituitary growth hormone (GH) is suppressed in acute stress forms (Pickering et al., 1991), while more prolonged forms tend to elevate GH levels (Sumpter et al., 1991a, b). Pituitary prolactin secretion is sensitive to handling and physical stress, although reported results appear to be somehow contradictory (Avella et

al., 1991; Pottinger et al., 1992). In most forms of environmental stress, pituitary adrenocorticotropin (ACTH) and other hormones of the pro-opiomelanocortin family are affected; melanocyte-concentrating hormone (MCH) are involved in response to handling and thermal shock. MCH modulates the stress response by suppressing the HPI activity. In physical forms of stress, such as handling and confinement, hormones of the pituitary-gonadal axis are suppressed (Pickering et al., 1987; Safford and Thomas, 1987). Testosterone and 11-ketotestosterone levels are suppressed in sexually maturing trout, *Salmo trutta* (Pickering et al., 1987). A similar androgen suppression was also noted in other teleost species (Safford and Thomas, 1987), a response that may be mediated by cortisol (Carragher et al., 1989; Avella et al., 1991). Environmental stress, acting via elevated cortisol levels, might also influence reproductive activity by suppressing gonadotropin synthesis/secretion by the pituitary gland.

Biochemical changes in relation to branchial and humoral adjustments, adopted by fish to maintain or increase oxygen uptake under stress conditions (Maxime et al., 1995), can be used as indicators of stress, culminating in an assessment of the use of adenylate energy charge (Houlihan et al., 1986; Bilik, 1990). In biochemical terms, these adaptive changes are immediately evident as an increase of haematocrit and total blood haemoglobin (Nikinmaa, 1983). The involved mechanisms are not completely understood, although it is known that stress can lead the inhibition of delta-amino levulinic acid dehydratase (ALA-D), an enzyme involved in the synthesis of hemoglobin. Suppression of ALA-D activity has been used as a specific indicator of lead poisoning in fish (Hodson, 1984). Fish subjected to severe hypoxia or prolonged exercise often exhibit a rapid depletion of muscle glycogen, the major source of energy under anerobic conditions (Van Waarde et al., 1983; Lennard and Huddart, 1992; Montpetit and Perry, 1998). The immediate consequence of a shift towards anaerobic metabolism is the tissue accumulation of lactic acid. Blood lactate levels have been used as a measure of the extent of anerobiosis and as indicator of stress in a variety of species (Waring et al., 1992).

Stress-induced hyperglycaemia occurs in several fish species in response to a wide range of stressors, including capture (Laidley and Leatherland, 1988), handling or disturbance (Schwalme and Mackay, 1985; Ellsaesser and Clem, 1987), emersion (Fletcher, 1984) and exposure to pollutants (Macfarlane and Benville, 1986).

Information on plasma amino acid and protein levels in stressed fish is limited (Vijayan and Leatherland, 1989; Ait-Aissa et al., 2003). Collagen, the most common animal protein, acts as an important structural element in all supporting tissues. Collagen deposition in the skeletal tissues of fish is markedly reduced in fish exposed to a variety of pollutants (Pavlov et al., 1990). The heat shock stress response is characterized by the induction of a set of stress proteins, namely heat shock proteins (HSPs). Due to their remarkable conservation throughout evolution, the wide diversity of inducing agents and the relative sensitivity of their expression in comparison to conventional endpoints, such as a growth, survival and reproduction, the HSPs, and especially the major stress protein HSP 70, have been proposed as sensitive markers of nonspecific effects during environmental monitoring (Ait-Aissa et al., 2003). Exposure of fish to certain pollutants can cause lipid peroxidation, a chemical process causing the oxidative deterioration of polyunsaturated lipids in biological membranes and ultimately leading to cellular damage (Thomas, 1990). Malondialdehyde is a breakdown product which has been used as an index of lipid peroxidation (Wofford and Thomas, 1988).

The exposure to xeno-estrogen is revealed by the presence of vitellogenin (Vtg) in the plasma of male fish (Sumpter and Jobling, 1995). However, more recent studies have also examined eggshell zona radiata protein (Zrp) production (Arukwe et al., 1998) or the expression of estrogen receptor, Zrp or Vtg mRNAs in liver samples (Yadetie et al., 1999).

Several lines of evidence indicate that RNA/DNA ratios are sensitive to different forms of stress and differences in the growth rate—as measured by changes in weight or length—can be correlated to this ratio (Kearns and Atchison, 1979). A lower ratio is detected in fish subjected to a variety of toxicants (Barron and Adelman 1985) or in larvae suffering from a diet-related intestinal disease (Steinhart and Eckmann, 1992).

Effects of stress on osmoregulation and ion balance in teleost fish were provided by Eddy (1981). Cathecolamine-mediated branchial and cardiac adjustments in stressed fish bring about ion loss in fresh water and ion inflow in sea water (Avella et al., 1991). Cortisol elevation may improve such changes by stimulating ion transport in both fresh water (Laurent and Perry, 1990) and seawater (McCormick et al., 1991).

Effects of acid exposure on the endocrine system of fish are reported by Wendelaar Bonga and Balm (1989). In fact, the problem of acid rain is

drawing attention on the impact of low pH on fish, especially in freshwater. A lowering in environmental pH simulating an acid episode, can cause catastrophic ion losses terminating in circulatory failure and death (Wood, 1989). In many situations, the combination of low pH together with elevated levels of metals can cause great damage to natural fish population (Reader and Dempsey, 1989). A study by Masson et al. (2002) demonstrated that plasma parameters in fish, such as Cl^- content, could serve as physiological indicators to evaluate water quality.

All forms of environmental stress can suppress the defense systems of fish to such an extent that susceptibility to disease is increased (Anderson, 1990). Much of the evidence implicates cortisol as an important factor responsible for this predisposition (Pickering, 1989).

The epidermis with its mucous layer functions as a protective barrier in fish. The external layer of mucus contains a wide range of bioactive molecules, such as immunoglobulins (Itami et al., 1988), lysozyme, chitinase (Lindsay, 1986), proteases (Braun et al., 1990), and hemagglutinins (Kamiya et al., 1988). Little is known about the effects of stress on mucus biochemistry. The activity of lysozyme, which also circulates in fish blood, declines after handling/transport stress or exposure to high ammonia levels; physical stress associated with emersion has been correlated with the occurrence of occult hemoglobin in the mucus of teleost fish (Smith and Ramos, 1976). Furthermore, if pathogens are able to penetrate the mucous/epidermal barrier, an inflammatory response may ensue, in which macrophages (primarily monocytes and neutrophils) migrate to the site of invasion to phagocyte any foreign material. This phagocytic activity can be measured by the degree of chemiluminescence resulting from the release of superoxide by stimulated macrophages (Pankhurst and Van der Kraak, 1997). Numerous assays have been set up to determine the degree of stress-induced immunosuppression in fish (see Andersson et al., 1988). Cytokines, variously termed lymphokines, monokines, interleukins and interferons, are important factors mediating many of the stress-induced changes in higher vertebrates (Andersson, 1990; Rivier, 1991). Studies in fish have shown that administration of recombinant mammalian cytokines have marked effects on the endocrine system (α-MSH release), epithelial function and liver metabolism (Balm et al., 1992).

Detoxification mechanisms involve several enzymes, particularly hepatic ones that have been proposed as ideal biochemical indicators of pollution (Jimenez and Stegeman, 1990; Goksøyr et al., 1996) The

enzymatic activities include antioxidants, such as catalase, superoxide dismutase and glutathione S-transferase (Gallagher et al., 2001; Peters et al., 2001), as well as other enzymes, such as ethoxyresorufin O-deethylase (EROD), aryl hydrocarbon hydroxylase (AHH), testosterone 6β-hydroxylase and fatty acid α-hydroxylase (FAH). They have been identified in numerous species of fish (Mathieu et al., 1991; Pesonen and Andersson, 1991; Ronisz et al., 1999) with sex-related (Gray et al., 1991) or seasonal (Lindstróm-Seppá, 1985; Jimenez and Stegeman, 1990; Mathieu et al., 1991) differences.

Little is known on the influence of non-pollutant environmental stress, although many xenobiotics, inducing cytochrome P-450 activities, can also stimulate the HPI axis of fish (Macfarlane and Benville, 1986; Mathieu et al., 1991; Goksøyr et al., 1996) and cortisol was found to potentiate the induction of various activities associated with the cytochrome P-450 enzyme system (Lemaire et al., 1994).

Metallothioneins have been proposed as biochemical indicators of heavy metal contamination (George and Olsson 1994; Goksøyr 1995). In fish, exposure to heavy metals promotes transcription of the metallothionein gene (Kille et al., 1992) and the elevation of tissue methallothionein levels is proportional to the degree of metal exposure (Hogstrand et al., 1991).

The synthesis of heat-shock proteins as a response to environmental perturbations is believed to increase tolerance of the cell to adverse environmental conditions (Kothay and Candido, 1982). Until further studies are undertaken, the importance of this response to stressed fish cannot be assessed (Ait-Aissa et al., 2003).

Social and environmental stresses may have deleterious effects on the reproductive function of spawners and on generational recruitment in fish populations (Fig. 20.3), though temporary mechanisms of resistance to stress have also been evidenced. Wingfield and Sapolsky (2003) suggest four mechanisms: (1) habituation at the central nervous system level (i.e. an individual no longer perceives the perturbation as stressful); (2) response attenuation at the level of the HPI axis (i.e., failure to increase secretion of glucocorticosteroids); (3) receptor down regulation at the level of the hypothalamo-pituitary-gonadal (HPG) axis (i.e., lowered response of the reproductive system to glucocorticosteroids); and (4) compensatory stimulation of the HPG axis to counteract inhibitory glucocorticosteroid actions. Although these mechanisms are likely to be

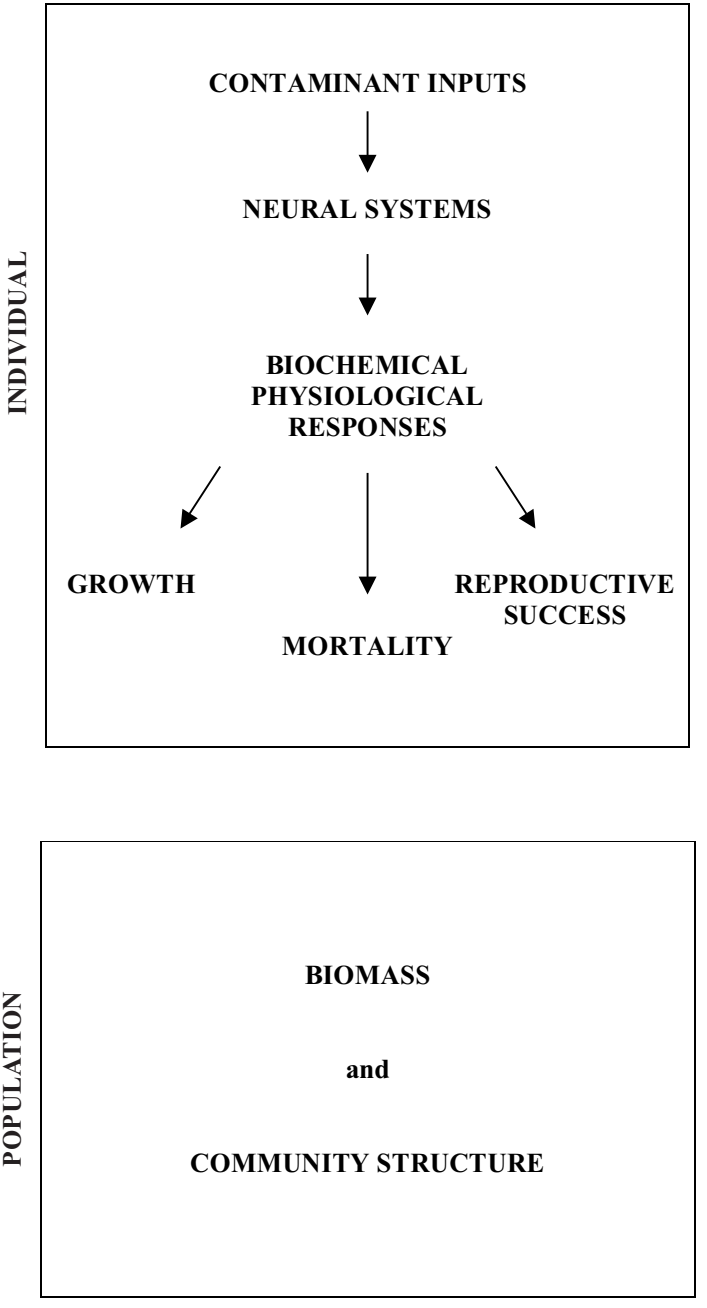

Fig. 20.3 Schematic representation of contaminant effects at different levels of organization on fish.

genetically determined, their expression may depend upon complex interactions with environmental factors.

During sexual maturation, stressed fish demonstrate altered levels of the circulating androgens, testosterone and 11-ketotestosterone (Pickering et al., 1987; Safford and Thomas, 1987). This effect can be mimicked by cortisol treatment of otherwise unstressed fish, thereby implicating the HPI axis in this response. Similarly, in maturing female fish, cortisol elevation causes a reduction of the circulating sex steroids, estradiol and testosterone, together with a decrease in plasma vitellogenin (Carragher et al., 1989; Haddy and Pankhurst, 1999; Kahl et al., 2001).

The cortisol-mediated suppression of female sex steroids can be demonstrated *in vitro* (Carragher and Sumpter, 1990) and cortisol treatment was found to reduce the number of estradiol receptors in the liver of rainbow trout (Pottinger and Pickering, 1990). In addition, the pituitary gonadotropin content is significantly decreased in cortisol-treated fish (Carragher and Sumpter, 1990). The depression of hypophyseal and gonadal activities in stressed fish results in a decrease of gamete quality with consequent reduced offspring survival (Campbell et al., 1992). The exact mechanisms of toxicant actions on fish reproductive function have not been fully elucidated, but they are known to include direct effects on steroidogenesis (Freeman et al., 1984), perhaps as part of a more general effect on lipid metabolism in the gonad (Kirubagarin and Joy, 1992), increased metabolic clearance of sex steroids due to the induction of steroid-transforming enzymes and, perhaps, of competitive inhibition on hormone receptors (Thomas, 1990).

STRESS AND REPRODUCTION

The defenses against stress in teleost fish reproduction show many similarities to those of other vertebrates. These concern the principal messengers of the brain-sympatho-chromaffin cell axis, equivalent of the mammalian brain-sympatho-adrenal medulla axis, and the brain-pituitary-interrenal axis, equivalent of the brain-pituitary-adrenal axis of tetrapods, as well as their functions, involving stimulation of oxygen uptake and transfer, mobilization of energy substrates, reallocation of energy away from growth and reproduction, and mainly suppressive effects on immune functions. There is also growing evidence for intensive interactions between the neuroendocrine system and the immune system in fish. Such differences, however, are present, and these are primarily related to the aquatic environment of fishes (Wendelaar Bonga, 1997).

Reproduction is variably affected by stressors in fish, in line with the great diversity of their environmental adaptations. If reproduction is to occur, then the fish must balance fecundity with gamete and progeny quality (Dauprat et al., 1990; Takahashi et al., 1998; Morehead et al., 2000; Schreck, 2000).

Teleosts have been shown to employ quite varied reproductive strategies to cope with stress (Kime, 1999). For example, stress seems to be involved in regulating sex reversal in hermaphroditic species. Shifts in reproductive tactics under stressful situations are probably important for fish in the wild to optimize reproductive fitness, and understanding of these phenomena is obviously important for the management of wild and hatchery stocks.

Reduction of reproductive performance is a common phenomenon associated with stress in vertebrates (Schreck, 2000), including fish (Barton and Iwama, 1991), because the physiological response of spawners to stress can have considerable consequence in terms of gamete quality and progeny viability (Dauprat et al., 1990; Takahashi et al., 1998).

It is well established that environmental variables can affect the timing of reproductive functions (Schulz and Goos, 1999). There is considerable plasticity for age and size of fish at sexual maturity in response to stress (Stearns and Crandall, 1984). Ultimate reproductive timing factors, particularly nutrition, can determine the age at first maturity and also age at and the frequency of subsequent reproductive events in fish (Gunasekera et al., 1995). A stressor that affects growth may lead to the production of a progeny that is already at a disadvantage because of its smaller size (Morehead et al., 2000).

The number of ripe eggs that a female produces is also based on environmental quality. Literature in this area deals with several topics, including: reproductive tactics relative to egg size, fecundity and age at maturity (Hislop, 1984); resource allocation for somatic growth and gonadal nutrient content (Encina and Granado-Lorencio, 1997); age-structuring, energy acquisition and fitness (Ware, 1984); the relation of hatching success to female condition (Laine and Rajasilta, 1999); and the importance of nutrients—particularly thiamine—in early embryo mortality syndrome (Hornung et al., 1998).

The timing of reproductive events, including puberty, atresia, gamete maturation, spermiation or ovulation, is influenced by seasonal, physiological variables responsive to stressors (Banks et al., 1999). The

physiology of gametogenesis and spawning appears to be tightly coupled with the stress physiology. Environmental variables, particularly nutrition, are eventually important in affecting gamete quality and reproductive timing (Wendelaar Bonga, 1997). The detrimental influence of poor feeding can be moderated by postponing puberty and/or by maintenance of quality of some eggs via atresia of the others.

Further integrative and comparative studies are required to appreciate the full range of variation in stress response among and within fish species; however, there is sufficient literature to suggest that different species can respond differently to similar stressors (Vijayan and Moon, 1994). Knowledge of the manner in which a stressor might affect the physiology of a species can help in developing management strategies that mitigate the impact of that stressor or even eliminate it through the use of an appropriate therapy (Schreck et al., 2001).

Antioxidants as Stress Biomarkers in Reproduction

Antioxidant systems scavenge and minimize the effects of free radicals and/or formation of oxygen-derived species (Eriksson, 1999; Klumpp et al., 2002; Pandey et al., 2003) in vertebrate reproductive processes (Maiorino and Ursini, 2002). Free radicals and reactive oxygen species play a number of significant and diverse roles in reproductive biology. In common with other biological systems, mechanisms have evolved to minimize the damaging effects that these highly reactive molecules can have on reproductive integrity. In particular, free radicals can cause germinal cell membrane modifications that impair fertilization (Marik, 2000) or zygote segmentation (Nars-Esfahani and Johnson, 1992). On the other hand, recent findings illustrate the constructive roles that oxygen radicals and reactive oxygen species play in a number of important steps during gametogenesis and the essential endocrine support they receive for the successful propagation of the species (Riley and Behrman, 1991; Winston and Di Giulio, 1991). Thus, reactive oxygen species production and antioxidant depletion by glutathione in mammalian germ cells are considered as necessary physiological events that are requisite to functional gametes maturation and to spermatozoa capacitation (Maiorino and Ursini, 2002).

Fishes, as other vertebrates, possess an antioxidant defense system, which utilizes enzymatic and non-enzymatic mechanisms (Wilhelm Filho et al., 1993; Peters and Livingstone, 1996). The more relevant antioxidant

defense enzymes are superoxide dismutase and catalase. Non-enzymatic defenses include the ubiquitous tripeptide glutathione, ascorbic acid, vitamin A, carotenoids, ubiquitol 10 and tocopherols (Rady, 1993; Pirihar et al., 1997; Pandey et al., 2003; van der Oost et al., 2003). Although current research interest extends to different classes of non-enzymatic antioxidants (Wilhelm Filho et al., 1993a, b; Roche and Bogè, 1996; Hai et al., 1997a, b; McFarland et al., 1999) and to antioxidant enzymes (Hai et al., 1997; Machala et al., 1998; Kolayli and Keha, 1999; Ritola et al., 1999; Zhou et al., 1999), the most important protection against the damaging effects of oxygen radicals is provided by a-tocopherol (vitamin E) (Di Mascio et al., 1991), essential in fish as in mammals for reproduction and post-natal development (Goodman-Gilman et al., 1992; Ciarcia et al., 1998).

Vitamin E and Reproduction: An Update

Tocopherols are widely distributed in animal tissues (Stocker et al., 1999), and are known to act as potent antioxidants in reproduction (Riley and Behrman, 1991). Their presence in biological membranes probably represents the major defense system against free radical attack and successive peroxidation of membrane lipids (Aten et al., 1994; Surai et al., 1998; Glascott and Farber, 1999). α-Tocopherol has been established as a radical chain-breaking antioxidant (Huo et al., 1996) that plays an important role in several biological processes (Guerriero et al., 2002). Unfortunately, its physiological mechanism of action still requires classification (Brighelius - Flohè and Traber, 1999). Experimental evidence exists on the different effects of vitamin E deficiency and its degree of severity in different animal species (Surai et al., 1998; Montero et al., 2001; Sahin et al., 2002). Previous studies on vitamin E determination in plasma (Hamre et al., 1997; Xue et al., 1998; Gieseg et al., 2000; Guerriero et al., 2002), gametes (Halliwell, 1999; Yamamoto et al., 1999; Marik 2000; Naziroglu et al., 2000), embryos and developing larvae (Sies, 1993; Campbell et al., 1994; Huo et al., 1996; Ionov, 1997; Surai et al., 1999; Ciarcia et al., 2000; Wang et al., 2002) confirm its predominant role as an antioxidant defense in many vertebrates (Halliwell, 1999). Indeed, different forms of vitamin E were analyzed in aquatic organisms such as artemia, rotifers, turbot, sea bass larvae and shrimp post-larvae (Huo et al., 1996; Wang et al., 2002). Moreover, vast literature exists on the role of vitamin E in the inactivation of sperm and

egg reactive oxygen species (Marik, 2000), and on semen quality and fertilization success (Grobas et al., 2002; Yousef et al., 2003).

Information is also available on the role of vitamin E against oxidative stress during embryo development (Campbell et al., 1994; Huo et al., 1996; Ionov, 1997; Surai et al., 1999). Vitamin E as a biomarker represents an index of the fish response to stress during reproduction.

Some aspects of larval development and sperm maturation and spermiation of the European sea bass, *Dicentrarchus labrax*, a widely utilized species in aquaculture, are specifically addressed in this review. The only available information was reported in our previous study (Guerriero et al., 2002). By means of HPLC analysis to titer the antioxidants in gametes and throughout larval development, we found high vitamin E levels in the plasma of adults of both sexes, in eggs before and after fertilization, in normal embryos and in embryos at hatching, in contrast to the low levels observed in dead eggs, dead embryos, and embryos with limited survival (Guerriero et al., 2004).

Vitamin E content in the yolk sac larvae decreased significantly during the first four days in both dead and living larvae ($P<0.05$). The vitamin E content of the dead yolk sac larvae at day 3 and 4 was not significantly different from that of vital yolk sac larvae (Ciarcia et al., 2000). In fact, during the first four days, the endogenous reserves of vitamin E, probably confined in the yolk sac, were rapidly utilized by the growing fish larvae, as also reported in other studies (Mani-Ponset et al., 1996). We hypothesized that, during organogenesis, there is an increased production of highly reactive and noxious free radicals that detoxificant mechanisms are not able to neutralize, as in other species (Umaoka, 1992).

In *D. labrax* prelarvae, the endogenous reserve of energy in the yolk sac is utilized in a few days (Mani-Ponset et al., 1996). In chick embryos, the vitamin E contained in yolk lipid droplets is taken up by the yolk sac membranes and processed into lipoprotein particles, which are released into the embryonic circulation (Surai et al., 1999). The temporal utilization of vitamin E in our samples was in agreement with the vitamin E utilization found in the developing chick embryo (Surai and Sparks, 2001). If vitamin E is not promptly replaced by feeding, larvae are not presumably able to neutralize reactive oxygen species and their survival is compromised (see Ciarcia et al., 2000). During larval development, vitamin E showed a further decrease from day 5—when the yolk sac is completely absent—attaining the lowest level at day 12 (data not shown).

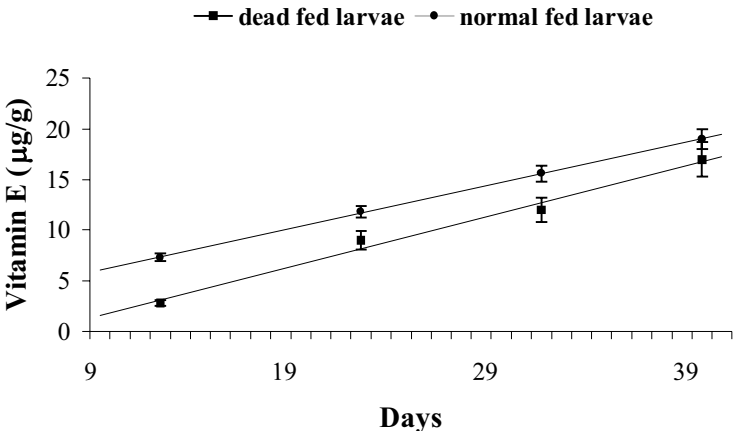

Fig. 20.4 Vitamin E content (mg/g dry weight) in normal (-●-) and dead (-■-) fed larvae of *Dicentrarchus labrax*, throughout the day 9 to 40 after hatching. Each value represents mean ± S.D. of three different determinations on 15 aliquots. (-●-) $r^2 = 0.99$; (-■-) $r^2 = 0.98$.

About 90% of unfed larvae died on day 12, whereas vitamin E levels steadily increased from day 9 to 40 in fed larvae (Fig. 20.4). The vitamin E content in dead fed larvae ($P<0.01$) is also reported in Fig. 20.4. Mortality during the first days of larval development was about 35% (Ciarcia et al., 2000).

Vitamin E did not significantly differ between normal and dead yolk sac prelarvae in the first five days, an indication that mortality was not dependent on depletion of the vitamin E content. Thus, mortality during the first days does not seem to be caused by a decrease of antioxidative endogenous defense (Ciarcia et al., 2000). Vitamin E requirement appears to be strictly correlated with an increase in metabolic rate and the consequent increment in the generation of reactive oxygen species (van der Oost et al., 2003). Hence, a vitamin E test would be recommended in fish farms as a possible marker of egg quality and fertility (Watanabe et al., 1985), since this vitamin is rapidly incorporated from the diet into the yolk (Meydani et al., 1992).

Gonads are known to have a high content of polyunsatured fatty acids on their cell membranes. This trait renders them prone to the deleterious effects of reactive oxygen species. Although excessive production may have deleterious effects, the controlled release and scavenging of some reactive oxygen species appears to modulate reproductive functions. Experiments were carried out on seasonal changes of vitamin E in the

testis of *D. labrax*, and variations were shown to be very similar to those found in plasma vitamin E levels (Guerriero and Ciarcia, manuscript in preparation). As a matter of fact, both testis and plasma vitamin E concentrations matched the plasma androgen levels, with a marked increase during gonadal recrudescence.

These data can be explained, like in mammals, with a specific role of vitamin E in steroidogenesis facilitation, tissue remodeling and synthesis of collagen, all events occurring along the testis-cycle (Riley and Behrman, 1991).

In conclusion, antioxidants as other biomarkers of stress can be detected and used to provide an early warning of potentially damaging changes in stressed adult fish.

CONCLUSIONS

This chapter briefly review stress biomarkers in fish, reports a biochemical antioxidative evaluation in cultured fish and proposes a role of antioxidants as stress biomarkers. It is a well-known fact that an antioxidant defense system neutralizes or limits stress effects in the reproduction of both fish and mammals. Specifically addressed has been the role exerted by vitamin E, the major antioxidant protector against reproductive damages during embryo and larval developments, and at the time of sperm maturation and spermiation of cultured sea bass. High vitamin E values were found in seminal fluid, in eggs before and after fertilization and in embryos during development and at hatching, whereas vitamin E was low in dead embryos or embryos with a limited survival. During larval development, the vitamin E content decreased slowly but steadily during the first four days after hatching; subsequently, it progressively increased from day 9 to 40. In adult male, testis and plasma vitamin E concentrations displayed a pattern in agreement with that of plasma androgen levels, with an increase during the gonadal recrudescence.

The indirect evidence of vitamin E as a biomarker of stress in sea bass reproduction supports previous studies in mammals. These observations also sustain the importance of antioxidants as biomarkers, critical for developing management strategies aimed to mitigate the impact of stressors and to implement appropriate actions in both aquaculture and fish conservation biology.

In accordance with other studies on stress defenses, we propose stress biomarkers as useful clues of stress response in fish, as claimed in other vertebrates. A better understanding of the role of stress biomarkers in fish reproduction will help limiting detrimental factors affecting broodfish well-being, gamete quality and progeny survival.

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CHAPTER

21

Neuroendocrine Mechanisms Regulating Stress Response in Cultured Teleost Species

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ABSTRACT

Neuroendocrine mechanisms regulating stress response in fish have been reported, with special emphasis addressed to ascertaining the stress-related effects on wild fish domestication and fish culture. The integrative network regulating stress response includes hormones produced by hypothalamus, pituitary, interrenal and gonads; those hormones influence fish metabolism, growth and reproduction. The neuroendocrine system was found to be able to modulate stress-response in terms of adaptation to environmental conditions in order to prevent the deleterious effects of stress, and to improve fish health, in the light of the modern organic aquaculture concepts, aimed both at fish safety, and that of consumers.

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Key Words: Fish; Stress; Neuroendocrine mechanisms; Stress response in farmed fish; Stress and feeding.

INTRODUCTION

Stress is followed by complex neuroendocrine responses: among these, the activation of the hypothalamus-pituitary-interrenal axis (HPI) and catecholamine release are fundamental for the adaptive response. The final goal of HPI axis activation is represented by the increase of cortisol plasma levels (Fig. 21.1). The interrenal release of cortisol is stimulated by pituitary ACTH, whose release into the blood stream depends on the action of the corticotropin-releasing factor (CRF). Indeed, CRF secretion from hypothalamic neurons increases upon stress stimulation. At the pituitary level, CRF stimulates the release both of beta-endorphin (β-EP) and beta-lipotropin (β-LPH), in addition to ACTH. All these peptide hormones represent the post-translational products of the common precursor proopiomelanocortin (POMC). The secretion of POMC-derived peptides in response to stress stimuli represents an important adaptive phenomenon. Every stimulus able to increase plasma levels of cortisol and cathecholamines could be considered a stress stimulus. Stress stimuli may be not only metabolic but also physical in nature. In addition to HPI axis hormones and catecholamines, other hormones such as prolactin (PRL) and growth hormone (GH) increase during stress. Moreover, exposure to chronic stressors reduces the reproductive function, probably involving hypothalamic GnRH release and/or gonadal function.

Teleost fish are, as other vertebrates, most sensitive to different kinds of natural stressors; in addition, several types of stress often occur in aquaculture conditions, when wild species are subjected to domestication. Indeed, the culture conditions interfere with homeostasis, and through the neuroendocrine system, cultured teleost species could switch on the stress response in terms of adaptation. This aspect is crucial in fish culture since the stress could counteract the efforts addressed to improving fish production and fish food availability. In fact, stress interferes with several functions, and the cross links between endocrine and immune systems could determine fish diseases and death.

Therefore, in this chapter, hormones involved in stress will be examined, and neuroendocrine stress-related responses will be discussed mainly in cultured teleost models; moreover, the new approach to fish

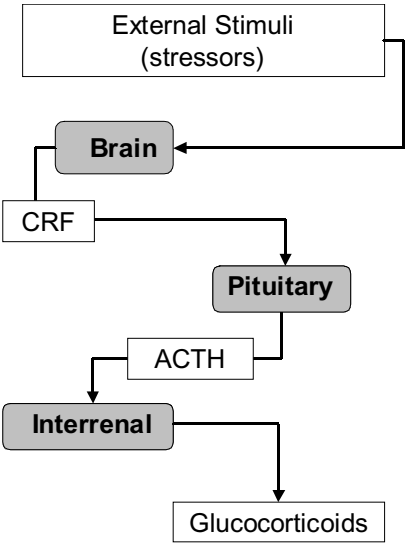

Fig. 21.1 Hypothalamus-pituitary-interrenal axis (HPI).

feeding in aquaculture to prevent stress-related effects will also be outlined.

STRESS AND HORMONES IN FISH

The major 'stress-related' hormones produced in, or released from the interrenal gland and the pituitary gland have been identified in fish as in every vertebrate class. In all cases, the secretion of these hormones increases in response to stressors. Acute stressors elicit an alarm (flight or fight) reaction by means of hypothalamic activation of the sympathetic nervous system. Corticotropin (ACTH) is released from the corticotropic cells of the pituitary gland pars distalis. ACTH is derived from the prohormone proopiomelanocortin, which is synthesized in the *pars distalis* and in the melanotropes of the *pars intermedia*. It is released mainly under the influence of the hypothalamic polypeptide, corticotropin-releasing-factor (CRF). ACTH then stimulates the release of interrenal steroids (cortisol). The interrenal response is associated with the general adaptation syndrome, in which the alarm reaction is followed by glucocorticoid secretion. Moreover, evidence exists that endogenous opioid peptides play an important role in stress response. Melanotropin (melanocyte-stimulating-hormone, MSH) is derived—in the form of

endorphin—from POMC; synthesized in the melanotropic cells of the pituitary neurointermediate lobe, MSH has been identified together with β-endorphin, as a possible stress-responsive hormone.

The generalized stress responses comprise physiological ones that are common to a wide range of environmental, physical and biological stressors; as indicators of a generalized stress response recently, several families of heat shock proteins (hsp) have been proposed (Iwama et al., 2004). Regarding environmental stressors, it seems of interest to mention the possible stress axis activation when the fish comes into contact—in both wild and culture conditions—with xenobiotics and, among them, chemical compounds that interfere with the endocrine system, namely endocrine disruptors (Eds). In Atlantic salmon, exposure to atrazine compromised their physiological capability to survive in saline conditions, and the surviving fish showed signs of major physiological stress, such as elevated plasma cortisol (Waring and Moore, 2004). Environmental estrogens, moreover, have been found to activate not only the feminization process in male wild aquatic species, but also the HPI axis, and to increase the level of peripheral glucocorticoids (Polzonetti-Magni et al., unpubl. data). Therefore, using appropriate molecular biology techniques, such as the DNA-array (Larkin et al., 2003), efforts are now being made to monitor the possible presence of Eds in the fish food chain. Because of their hydrophobicity, Eds bioaccumulate in the animals and produce deleterious effects by activating several gene expressions. In fact, modern aquaculture is currently being addressed to the certification of products, such as organic ones, since the principles behind organic aquaculture closely mirror those for land-based practices, which aim to promote sustainable systems of food production and high standards of livestock welfare (Fairbrother, 2004).

It should also be pointed out that in cultured fish, a variety of stressors are known to influence secretion of prolactin (PRL) (Pottinger et al., 1992), somatolactin (Kakizawa et al., 1995; Zhu and Thomas, 1995), and IGF-I (Dyer et al., 2004). Moreover, stress response also activates changes in gene regulation that may play an important role in adaptation (Schulte et al., 2000; Picard and Schulte, 2004; Sardella et al., 2004, Sarrofoulou et al., 2005).

Role of Cortisol During Stress

Cortisol is the principal corticosteroid in teleost fishes and its concentration rises dramatically during stress; elevated cortisol levels are

responsible for increasing blood glucose concentration in response to the energy demand needed for the increase of metabolic rate and oxygen uptake in stressed fish. The mechanisms of action, and metabolic regulation of cortisol in teleost have been widely reviewed by Mommsen et al. (1999) who discussed this hormone not only as an essential component of stress response, but also in view of its significant role in osmoregulation, growth and reproduction. Thus, cortisol promotes processes essential for adaptation to stressors and, consequently, cortisol levels have been considered a valuable stress index (Ven der Salm et al., 2006).

In this context—it must be pointed out that in chronic stress, which often occurs in cultured fish—the increased plasma glucose levels produce deleterious effects on fish health (Pickering, 1989).

Various types of stress occur in aquaculture conditions; temperature, changes in salinity for marine teleost species, crowding, netting, photoperiod and quality of water, such as the presence of toxicants, can dramatically alter fish homeostasis, and the duration of such conditions dramatically influences growth rate, reproduction performance, and the immune system (Pickering and Pottinger, 1987, 1989; Pickering, 1989, 1992). Therefore, the effects of various stressors and their duration were described in teleost species, in which cortisol measurement was related with the stress response.

In rainbow trout, a freshwater fish widely cultured for several decades, ontogeny of the cortisol stress response in larvae was described by Barry et al. (1995a) showing that the hypothalamic-pituitary-interrenal (HPI) axis first develops responsiveness to acute stress two weeks after hatching and one week before the onset of exogenous feeding. Activation of the HPI axis, resulting in transiently or chronically increased plasma cortisol levels, has also been related with the changes induced in the epithelial tissues. Those effects were described in several works by Iger (1992) in the carp, then in the trout (Iger et al., 1995), in which cortisol in the diet transiently elevates plasma cortisol levels alongside profound and prolonged adverse changes in the skin, suggesting a cortisol-mediated response in these animals.

Cortisol release during exposure to stressful conditions has an adaptive role in the short term, but when cortisol levels are elevated over a prolonged period cortisol may increase susceptibility to disease, depress growth rate and interfere with reproduction (Pickering, 1992). During prolonged exposure to a stresssor, when a number of mechanisms may help to reduce the deleterious effects of elevated cortisol levels, there is

considerable inter-individual variability depending on a genetic basis (Fevolden et al., 2002). To offer the potential for optimizing performance of fish under intensive rearing conditions in aquaculture, lines of rainbow trout with low- and high-cortisol response to stressors have been produced by selective breeding (Fevolden et al., 1993, 2002; Pottinger and Carrick, 1999). Elevated cortisol in the blood has also been found as a consequence of stress response to hypoxia in parrot fishes inhabiting the coral reefs (Turner et al., 2003), in Nile tilapia, as an effort of the establishement of dominance (Correa et al., 2003), and in goldfish, a hearing-specialist fish, susceptible to noise-induced stress and hearing loss (Smith et al., 2004).

Through negative feedback loops at every level of the HPI axis (Sumpter, 1997), cortisol also plays an important role in preventing the adaptive features of the endocrine stress response to threatening and overshooting homeostasis. However, despite the regulatory role of cortisol in limiting the size of the stress response, chronic stress can be detrimental to fish and negatively inhibit various aspects of performance, including growth (Barton and Iwama, 1991).

Although the effects of chronic stress on growth are not always paralleled by sustained increases in plasma cortisol levels (McCormick et al., 1998), available evidence suggests that cortisol is a primary mediator of the growth-suppressing effects (Pickering, 1993; Pankhurst and Van der Kraak, 1997), and that chronically elevated plasma cortisol decreases growth (Barton et al., 1987; De Boeck et al., 2001). The physiological and biochemical changes responsible for the growth-suppressing effects have been widely reviewed by Mommsen et al. (1999). In brief, through the mobilization of stored energy and an increase in gluconeogenesis, cortisol may divert energy from the anabolic processes (Vijayan et al., 1993, 1997; De Boeck et al., 2001:). Moreover, it has been found that excess cortisol in goldfish can be associated with poor growth despite normal food intake, with the forebrain NPY and CRF (corticotropin-releasing-factor) playing a role in mediating that effect (Bernier et al., 2004).

Cortisol is a very important hormone showing in fish both a corticosteroid and a mineralcorticoid function; it is synthesized in the interrenal tissue, a diffuse tissue located in the head kidney, which is equivalent to the mammalian adrenocortical gland. The mechanisms underlying cortisol synthesis are consistent with the knowledge that ACTH signal transduction involves cAMP (Patino et al., 1986), as well as cAMP-dependent protein kinase A and C (Lacroix and Hontela, 2001). The activity of steroidogenetic enzymes has been evaluated (Colombo et

al., 1972; Balm et al., 1989; Sangalang and Uthe, 1994), as well as the characterization of the enzyme mRNAs involved in the metabolic pathways of interrenal tissue, also described using molecular biology approaches (Liu et al., 2000; Govoroun et al., 2001). The expression of the genes involved in cortisol synthesis in rainbow trout was then examined in response to two different acute stressors, and an acute ACTH treatment was recently performed by Geslin and Auperin (2004); in that study, mRNA levels of the StAR (steroidogenetic acute regulatory) sterol transport protein, which transports cholesterol to the inner mitochondrial membrane, and, as well, cytochrome P450 cholesterol side chain cleavage (P450 sec) were determined in head kidney (containing the interrenal tissue); it was found that the high levels of cortisol after stress need an activation of genes involved in cortisol synthesis, but lower levels do not, suggesting other types of regulatory mechanisms in cortisol production.

Secretion of Proopiomelanocortin-derived Peptides in Stress Response

Proopiomelanocortin (POMC) is a precursor of a number of peptides that can be divided into three groups: adrenocorticotropic hormone (ACTH)-like, endorphin-like and MSH-like products (Fig. 21.2). Post-translationally, POMC-derived peptides can be modified, for example by glycosilation and acetylation. In all vertebrate classes, both corticotrophs of the pars distalis (PD) and the melanotrophs of the neurointermediate lobe (NIL) of the pituitary gland synthesize a common precursor molecule, POMC. However, the processing of POMC differs in the two cell types: in the corticotrophs, the final products are predominantly an N-terminal peptide, adrenocorticotropin (ACTH), and β-lipotropin (β-LPH); some of the β-LPH may be further processed to β-LPH and endorphin. Processing of POMC proceeds in the melanotrophs, leading to β-melanocyte stimulating hormone (β-MSH), corticotropin-like intermediate lobe (CLIP), β-MSH and endorphin, which are all major final products (see Kawauchi, 1983, for review of fish). In fish, as in other vertebrates, the function of corticotrophs is to stimulate corticosteroid secretion from interrenal glands by means of ACTH in response to stress.

The function of the melanotrophs, at least in lower vertebrates, is the regulation of color changes by means of melanotropin secretion. In different types of stress paradigms, the stress response of corticotrophs and melanotrophs has been found to be dependent on the type of stressor and

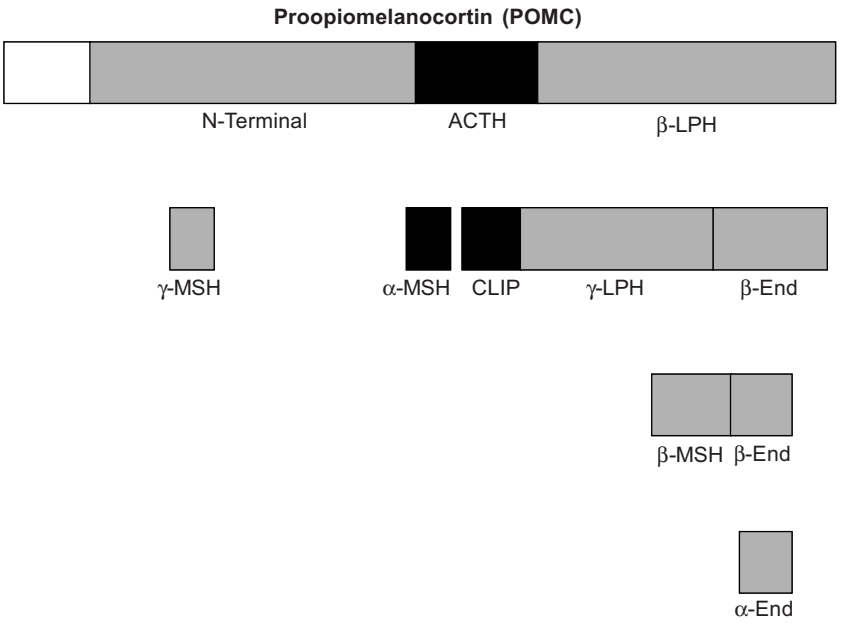

Fig. 21.2 Opioid peptides derived from proopiomelanocortin (POMC) cleavage.

its duration (Sumpter et al., 1985; Lamers et al., 1991), while corticotrope/melanotrope POMC-derived peptides are related to interrenal function during stress in rainbow trout (Balm and Pottinger, 1995). In tilapia, the melanotrophs have been found to be potent regulators of interrenal response, although they may not be operative under all conditions (Balm et al., 1995); moreover, tilapia may be able to modulate not only the quantitative but also the qualitative signal from the MSH cells (Lamers et al., 1992; Balm et al., 1995).

The knowledge so far available on the role of POMC-derived peptides in stress-response in fish is consistent with the evidence that stressors activate the HPI axis, and the subsequent increased release of POMC-derived peptides from the pituitary gland induces cortisol release from the corticosteroid-producing cells of the head kidney (Fig. 21.3). A variety of external stimuli is known to induce stress response in fish (for a review, see Wendelaar Bonga, 1997). In fact, in response to changes in ambient temperature, POMC genes are expressed in common carp and their expression is also strain-dependent (Arends et al., 1998). Moreover, enzymes responsible for the proteolytic cleavage of POMC, the prohormone convertase, have been found in fish (Roth et al., 1993). The

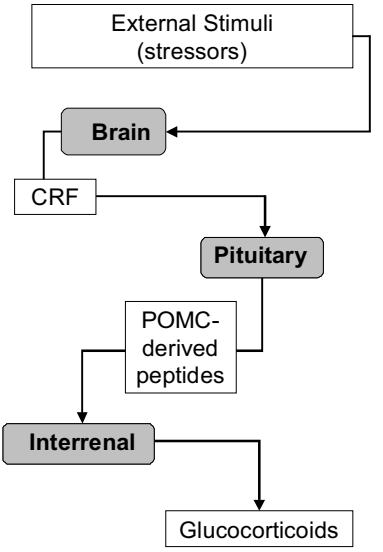

Fig. 21.3 The hypothalamus-pituitary-interrenal axis and POMC-derived peptides.

convertases have been identified as members of the subdivision family of serine endoproteases, which have been found also in protochordates (Oliva et al., 1995; Kawahara et al., 2003).

Proopiomelanocortin-derived peptides have been found present not only at central levels but also in peripheral organs; their involvement in neuroendocrine-immune communication is also known (see Stefano et al., 1996), and opioid peptides involved in the stress response are present in the gut from the early larval stage of sea bass (Mola et al., 2004), and in other teleost species (Rombout and Reinecke, 1984; Barrenechea et al., 1994). Moreover, opioids are also involved in reproduction because of their colocalization with decapeptide gonadotropin-releasing-hormone (GnRH) at hypothalamic level, where both regulate gonadotropin secretion (Chieffi et al., 1991).

POMC-derived peptides have also been identified in reproductive organs: in the ovary of seabream *Sparus aurata* and sea bass, *Dicentrarchus labrax*, MSH-like and β-EP-like peptides were identified and measured; the changes of MSH were found related with reproductive function, and the amount was higher in fish-farmed animals compared with that found in wild ones.

Besides with reproductive function, the changes of ovarian melanotropic peptides have been found also related with environmental

factors, such as natural conditions and/or ecophysiological manipulation of the day length and temperature adopted in the fish-farm; in fact, β-EP-like peptide was found significantly higher in wild than in farmed seabream and sea bass (Carnevali and Mosconi, 1998, for review).

Therefore, the POMC-derived peptides both at central and peripheral levels, functon as 'alarm-peptides' under different environmental conditions (Fig. 21.4).

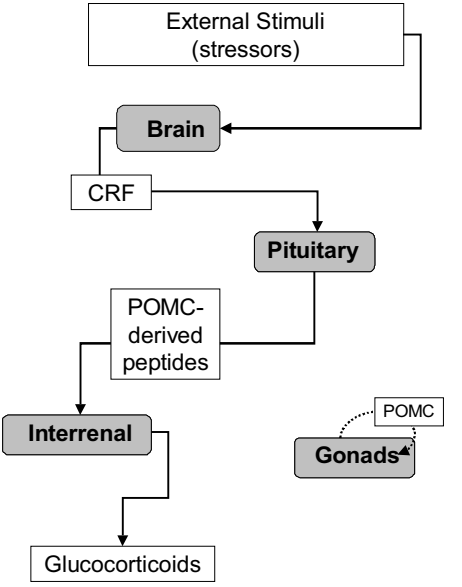

Fig. 21.4 Stress-related response through the activation of glucocorticoids and gonadal opioids.

Reproductive Hormones and Stress

The reproductive function is regulated through long-loop feedback mechanisms, the hypothalamus-pituitary-gonadal (HPG) axis, and local cellular signaling (Fig. 21.5). The hypothalamic neurohormone, the decapeptide gonadotropin-releasing-hormone (GnRH), stimulates the synthesis and release of pituitary gonadotropins which, in turn, modulate steroidogenesis and gametogenesis functions of the ovary and testis. In female fish, GTH-I plays a role in inducing estradiol secretion regulating vitellogenesis, and GTH-II seems involved in oocyte maturation, a process well described by Nagahama (1994). In male fish, the interstitial Leydig

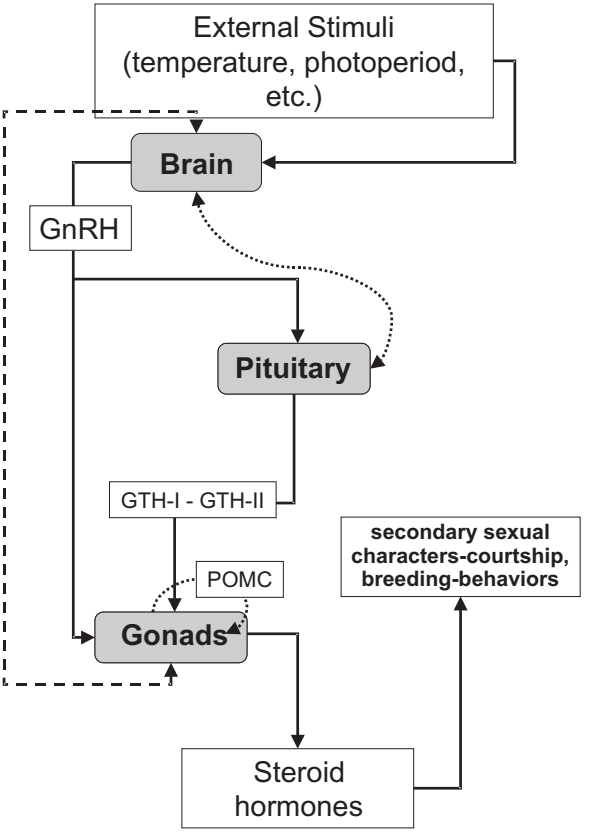

Fig. 21.5 Hypothalamus-hypophysial-gonadal axis regulating reproduction through both long- and short-loop feed-back mechanisms.

cells produce steroids, especially androgens, which regulate spermatogenesis. Stressors may affect the hypothalamic components of the male and female reproductive system by altering GnRH function, which results in decreased synthesis of gonadotropins. The effects of stress on the HPG axis are influenced by the type and duration of the stimulus and in several vertebrate species the increase of peripheral corticosteroids is accompanied by the decrease of peripheral sex steroids (Mosconi et al., 1994b; Welsh et al., 1999).

The capture, handling, crowding, and bleeding of fish may constitute a stress and affect circulating hormone levels (Carragher and Sumpter, 1990). Cortisol is a major 'stress-related' hormone, but besides corticosteroids, also the secretion of sex steroids is influenced by stress in fish and in other vertebrates.

Androgens are decreased by stress in mammals (Welsh et al., 1999), reptiles (Moore et al., 1991) and amphibians (Licht et al., 1983; Mosconi et al., 1994b). In salmonid fish, an inverse relation between cortisol and androgen levels has been found during smoltification and sexual maturation (Pickering et al., 1987). It has also been reported that exogenous cortisol in teleost fish affects reproductive function and gamete development (Carragher et al., 1989; Carragher and Sumpter, 1990; Foo and Lam, 1993; Barry et al., 1995b).

The effects of acute stress on plasma cortisol, sex steroids and glucose levels were described in male and female salmon (*Onchorynchus neka*), during the breeding season (Kubokawa et al., 1999). Acute stress increased cortisol and glucose levels in males and decreased sex steroid levels, while female salmon showed only the decrease of sex steroids, suggesting a sex-response to stress in this species.

In farmed species, reproduction is a key event by which eggs of good quality, larvae and juveniles must be produced to improve rearing activities, increase the fish market, and restock wild populations; therefore, the fact that stress induces deleterious effects in broodstock domestication and reproductive performance must be taken into account.

FISH CULTURE IN THE MEDITERRANEAN AREA

The production of marine fish in the Mediterranean region has recently undergone exceptional development, too, due both to the decline of freshwater fish species production and a shift to that of marine fish undoubtedly related to a growing demand for high-quality fish by the European and the Mediterranean consumers (Gouveia, 2003). The production of fish in the Mediterranean region is a traditional activity that originated in ancient times, where its earliest evidence dates back to the pharaonic period in Egypt (2500 BC). More recently, in the fifth century BC, shellfish production was reported to be practiced by the Greeks, and in the sixth Century BC, there was evidence of marine fish production by the Etruscans (Ferlin and LaCroix, 2000). Common carp, the earliest farmed fish, was introduced into Europe during the Middle Ages for culture in monastic ponds (Pillay, 1990). So, beyond any doubt, the production of fish in the Mediterranean region started early and its beginning remains unknown, perhaps, lost in the mist of time.

Currently, marine fish production in the Mediteranean area consists not only in the culture of sea bass, *Dicentrarchus labrax*, and the gilthead

seabream, *Sparus aurata*, but also that of new species has been attempted, in order to diversify the market, and to provide certified species produced according to standards of organic fish farming, which include stocking densities, feed ingredients, disease and pesticide management (Fairbrother, 2004). These standards are closely related with the very common adverse factors that stress induces in fish and, in turn, with fish food of low quality in terms of consumer health.

The neuroendocrine stress axis activation will be presented in the traditional marine teleost species, sea bass and seabream, and in a new species, the sole, a marine teleost of a great commercial interest, whose culture has been recently attempted in the Mediterranean area (Mosconi et al., 2001).

Neuroendocrine Stress Axis in Cultured Marine Teleost Models

The proopiomelanocortin (POMC) gene, which encodes the common precursor for MSH-related and β-endorphin-related end products, appeared early in chordate evolution and features a variety of lineage-specific modifications, extensively described in the work of Dores and co-workers (Danielson and Dores, 1999; Danielson et al., 1999). In fish, the presence of the POMC system is extensively documented both in elasmobranchs (Vallarino and Ottonello, 1987; Vallarino et al., 1989b; Amemiya et al., 1999; Chiba, 2001), and teleosts (Kawauchi et al., 1980; Vallarino, 1985; Vallarino et al., 1989a; Dores et al., 1993). A great deal of research has been conducted on the responses of fish to stress (Pickering, 1981), and on the involvement of POMC-derived peptides in the modulating of stress responses by activation of the HPI axis (Van der Burg et al., 2005). In salmonids, handling and confinement has only activated the corticotrophs of the pars distalis, and not the melanotrophs of the neurointermediate lobe, whereas when the handling was combined with thermal shock, both cell types were activated (Sumpter et al., 1985, 1986). In addition, in tilapia, three forms of α-MSH (des-acetyl, mono-acetyl, and di-acetyl α-MSH) have been identified in neurointermediate lobe and plasma; the peptides are acetylated intracellularly and tilapia may be able to modulate not only the quantitative but also the qualitative signal from MSH cells, enhancing the flexibility of the animal to respond to environmental challenges.

Regarding sea bass and seabream, melanotropic peptides have been found present not only at the central level, but also in the ovary, playing a physiological role in the interaction between reproductive function and environmental cues (Mosconi et al., 1994c). In addition, their ovarian content was related with environmental factors, such as natural conditions and manipulation of day length and temperature adopted in the fish-farm. The different ratio between α-MSH and ACTH (1-13) amide (des-acetyl-α-MSH) in reproductive wild sea bass and seabream, in comparison with those taken from the fish-farm, strongly support the role played by melanotropic peptides in adaptation. As in tilapia, in sea bass and seabream also, the different forms of melanotropic peptides may be able to modulate the signal in response to stress (Mosconi et al., 1994c). Moreover, in the same species, both opioid systems—peripheral and central—are operative, and their level of activity was found to be related to stress responsiveness. In the ovary of sea bass and seabream, an acetylated peptide similar to the chum salmon N-acetyl β-endorphin II peptide (act-sEP) was found, its content being much lower than in the pituitary. Nothwithstanding, the two opioid systems appear to be coordinated since pituitary endorphins act systematically and those in the ovary presumably in a paracrine fashion; moreover, act-sEP was found to be consistently much higher in both ovary and pituitary of wild fish in comparison with the farmed ones (Mosconi et al., 1994a). Such a difference may depend on the greater sensitivity to stress stimuli and reactivity to stressors of wild fish compared with that found in domesticated strains, since the magnitude of stress reaction is a hereditable character in fish, and the lower stress reactivity of domesticated stocks is likely due to unplanned mass selection by the fish farmers. In fact, by selecting as brood-stock the fish with the best growth, the farmers are induced to also select fish that have suffered less from stress (Colombo et al., 1990). The greater amounts of an endorphin in acetylated form—hence incapable of binding to its cognate receptor—support the view that opioid systems are more activated in wild than farmed fish, as shown also by Woodward and Strange (1987) for the HPI axis and the sympatho-chromafin system. Moreover, acetylation/deacetylation reactions may provide a regulatory mechanism to prolong the endorphin action of this 'alarm' peptide, allowing a more sustained response to stress. Then, the stressor-specific response in cultured male seabream was investigated together with the relations between opioid and HPI systems. Two types of stress paradigms that may occur in fish-farm

conditions were applied; after long-term confinement and crowding, short-term confinement, crowding and manipulation, plasma cortisol and act-sEP, as well as pituitary act-sEP, content were measured (Mosconi et al., 1998). In long-term confinement and crowding, higher plasma cortisol levels and act-sEP contents than in the control group were found to be well correlated. However, although plasma cortisol increased in both types of stress paradigms, a significant increase of plasma act-sEP was found only in the case of confinement and crowding, suggesting a direct correlation of act-sEP exclusively in cases of specific stress, and supporting the different nature of the pituitary-interrenal stress response. In the short-term stress experiments, the double activation of both opioid and corticotrope systems was ascertained. Moreover, treatment with naltrexone—an opiate receptor antagonist—supports the idea that the pituitary-interrenal response could be endorphin-dependent. Therefore, in this marine teleost model, the activation of the opioid system is stressor-specific and this system, namely act-sEP, regulates the activation of the pituitary-interrenal axis during stress conditions, and endorphin could thus be considered a signal peptide for adaptation from wild to fish-farm conditions (Fig. 21.6).

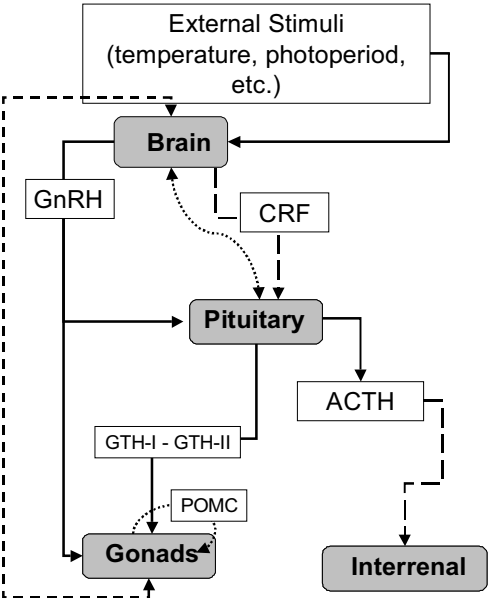

Fig. 21.6 Scheme depicting the integrative network regulating stress-response in fish.

The activation of the HPI axis was also found in *Solea solea* juveniles during three weeks of crowding stress; plasma cortisol levels significantly increased in the first week, but then slightly decreased levels were found at the end of the experiments when, besides the cortisol trend quite similar to that of control, the measurement of growth performances revealed deleterious effects of such a stress paradigm on the growth rate of sole juveniles (Cardinaletti et al., personal communication).

The depletion of flatfish fisheries as well as the continuing and strong consumer demand for high-value flatfish have affected aquaculture efforts worldwide. The success of aquaculture ventures requires the optimization of growth and the health of the fish at all life history phases (Waters, 1996). Substantial progress has been made in a number of areas, such as larval nutrition and the control of spawning (Dhert et al., 1994; Gara et al., 1998; Mangor et al., 1998; Ronnestad et al., 1998; Dinis et al., 1999). The domestication of a broodstock of *Solea solea* was performed in the Mediterranea area (Tyrrhenian Sea, Orbetello Pesca Lagunare): spawning induction through ecophysiological manipulation has been successfully attempted, and production of larvae and juveniles been obtained (Cardinaletti, pers. comm.). Nevertheless, fundamental aspects of sole development, and development defects that commonly occur in hatcheries, have happened, such as malpigmentation, particularly albinism (hypomelanosis). Albinism reduces the market value of hatchery reared fish; albino juveniles lack cryptic coloration and are, therefore, easily visible to predators in natural environments. This contributes to poor survival rates when the hatchery raised fish are used to supplement wild stocks or enhance coastal fisheries (Furuta, 1998; Furuta et al., 1998). In fact, to supplement sole wild stocks was one goal of the project set up in the Tyrrhenian fish-farm. The problems related with pigmentation development in hatchery-reared flatfishes have been reported by Venizelos and Benetti (1999), and widely reviewed by Bolker and Hill (2000). Regarding the sole reared in the Tyrrhenian fish farm, it was hypothesized that larval albinism, consisted in the possible effects of crowding stress on abnormal pigmentation related with the activation of the HPI axis; therefore, besides the explanations given by researchers (Bolker and Hill, 2000), it seems that neuroendocrine stress response, through opioid system activation, must be investigated in greater depth.

FISH FEEDING IN AQUACULTURE TO PREVENT STRESS-RELATED EFFECTS

Among several critical points in the cultivation of fish, reproduction, spawning, growth performances, and suitable aquafood, the most critical period for several marine species is the larval stage; the mass mortality during larval rearing is commonly caused by bacterial infections. Bacterial diseases can also occur when not very well-defined rearing conditions induce stress response which is deleterious in decreasing the immune system, and the capability of fish to resist infections. Currently, either treatment with chemotherapeutic agents or vaccination is used to protect fish against different bacterial diseases in hatchery conditions. However, extensive use of antibiotics in aquaculture disease control leads to resistant bacteria. For this reason, their use represents an ecological threat to coastal areas exploited for industrial cultivation of fish and shell-fish, and thus it should be restricted.

Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the larval rearing tanks. One such method gaining acceptance within the industry is the use of probiotic bacteria to control potential pathogens. Probiotics are usually defined as live microbial feed supplements, administered in such a way as to enter the gastrointestinal tract and to be kept alive, and which beneficially affect the host animals by improving their intestinal microbial balance and, in turn, the host health (Gatesoupe, 1999). Several bacteria have recently been used as probiotics in the larval culture of aquatic organisms (Timmermans, 1987; Sissons, 1989; Gildberg, et al., 1997; Vadstein, 1997; Ringo and Gatesoupe, 1998; Hansen and Olafsen, 1999; Gomez-Gil et al., 2000). More recently, live food was employed as a vector of probiotic bacteria in seabream and sea bass post-hatching development (Zamponi et al., 2003; Carnevali et al., 2000), by using as a vector both rotifers and artemia. Extremely encouraging results were obtained since in the probiotic-feeding group, significant decrease of stress-related parameters and mortality were found together with an improving of GALT (gut-associated lymphoid tissue); the latter indicates the role of probiotics in potentiating the immuno system in larval stress responses.

Probiotic larval feeding could be also considered as an important tool in sole rearing to counteract the abnormalities in pigmentation; a problem of abnormal coloration was found also in hatchery reared Japanese flounder, where normal body coloration was achieved in the larvae fed

with *Artemia* enriched with an appropriate dose of vitamin A (Dedi et al., 1995).

The Potential Role of Cortisol in Regulation of Food Intake

Elevated plasma cortisol is—as previously reported—a key event of the endocrine response to stress in fish and results from a stimulation of the HPI axis. In response to a variety of stressors, cortisol contributes to the mechanisms involved in maintaining homeostasis primarily by mobilizing energy to meet the increased metabolic demand (Mommsen et al., 1999). Despite the regulatory role of cortisol in limiting the size of the stress response, chronic stress can be detrimental to fish and have a negative impact on various aspects of performances including growth (Barton and Iwama, 1991). Although the plasma cortisol levels do not seem to be always related with growth rate in stressed fish, considerable evidence suggests that cortisol is a primary mediator of the growth-suppressing effects of stress, mainly by chronically elevating plasma cortisol (Barton et al., 1987; Pickering, 1993; Pankhurst and Van der Krak, 1997; De Boeck et al., 2001). The question is whether the growth-suppressing effects due to elevated cortisol depend on the reduction in food intake or on the reducing absorption of food through the intestine (Mommsen et al., 1999), since reduction in appetite has been shown to be a characteristic feature of the behavioral response to stress (Schreck et al., 1997).

The regulation of food intake in fish, as in other vertebrates, appears to be achieved through a complex hypothalamic network that integrates orexigenic (stimulatory) and anorexigenic (inhibitory) neuroendocrine signals of central and peripheral origin (Lin et al., 2000; De Pedro and Bjornsson, 2001). It has been suggested (Bernier et al., 1999) that cortisol might interact with the appetite regulatory pathways through its negative feedback on the forebrain expression of corticotropin releasing factor (CRF). In addition, CRF is both the hypothalamic regulator of the HPI axis (Lederis et al., 1994) and a potent anorexigenic agent in goldfish (De Pedro et al., 1993, 1997; Bernier and Peter, 2001); in this species, Bernier et al. (1999) provided evidence on negative feedback regulation by cortisol of CRF gene expression. Moreover, in salmonids, CRF may have an important role in the control of stress responses (Ando et al., 1999), and in tilapia CRF is involved in the regulation of stress-related peripheral processes; unstressed tilapia had undetected CRF levels and their CRF

levels increased after acute stress, whereas this increase was absent after chronic stress (Pepels et al., 2004).

The effects of cortisol on food intake have been recently and widely investigated by Bernier et al. (2004) in goldfish, showing that while moderate increases in plasma cortisol can stimulate food intake slowly over several days, larger doses of glucocorticoids may mask the appetite-stimulatory effects of cortisol. Thus, excess cortisol can be associated with poor growth despite normal food intake, and forebrain neuropeptide Y and CRF play a role in mediating the effects of cortisol on the food intake.

In mammals, the CRF system is also found to be a major modulator of integrated physiological responses to stress. At the hypothalamic-hypophysial level, it exerts a potent stimulation of ACTH, and its localization suggests that CRF neurons are involved in the regulation of autonomic stress response (Valentino et al., 1991). Studies conducted in both human and laboratory animals have demonstrated that dysregulation of the CRF system is implicated in a variety of psychiatric disorders. In this context, Ciccocioppo et al. (2001, 2003) discovered that an opioid peptide structurally related to dynorphin A, the nociceptin/orphanin FQ (N/OFQ), inhibits stress- and CRF-induced anorexia in rats; it binds selectively to its receptor, referred to as the opioid N/OF2 receptor (NOP), receptor-like opioid. N/OF2 has an opposite effect to that of CRF, and an NOP receptor agonist completely blocks the anorexia effect induced by stress or CRF (Ciccocioppo et al., 2001, 2002).

Efforts in studying stress in fish should be addressed to identifying the possible function of this opioid peptide in stress-related effects on food intake, since N/OF2 related gene was found in teleost species (Danielson et al., 2001).

FUTURE CHALLENGES

In this chapter, the neuroendocrine stress-response in fish was considered to be a mechanism by which stressed fish can positively modulate their homeostasis. The role of hormones and neurohormones involved in stress were discussed with major emphasis being laid or glucocorticoid, cortisol metabolic effects and those exerted by the opioid system.

The most important aim was to encourage scientific efforts in this field, since fish culture needs more basic knowledge on fish management, which includes several activities related with aquaculture practice.

The suggestion is to follow new ways of investigation now becoming possible by applying genomic and proteomic approaches.

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CHAPTER

22

The HPA Axis and Functions of Corticosteroids in Fishes

David O. Norris* and Steven L. Hobbs

ABSTRACT

In this chapter, we shall discuss the nature and operation of the neuroendocrine axis that controls the activity of the fish adrenocortical tissue (interrenal gland). We will also consider the roles of corticosteroids in ionic and osmotic balance, metabolism, and stress as well as the effects of environmental contaminants on the system. Finally, we will address the general roles of corticosteroids and their receptors in development, reproduction, and aging.

Key Words: Corticosteroid; Glucocorticoid receptor; Mineralocorticoid receptor; Iono-osmotic regulation; Metabolism; Immunity; EDCs; Aging; Development; Reproduction; Adrenocortical tissue; Interrenal; Stress; HPA axis; HPI axis.

THE HPA AXIS IN FISHES

In mammals, the major neuroendocrine regulatory system consists of the

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hypothalamus in the brain, the nearby pituitary gland or hypophysis, and other endocrine glands that are regulated by pituitary secretions: the thyroid, (see: Eales, this volume) the adrenal cortex, the gonads (testes and ovaries) (see: Signer et al. this volume) and the liver (see Norris, 1997). In the hypothalamus, specialized neurons, called neurosecretory (ns) neurons, secrete neuropeptides called releasing hormones into a system of portal blood vessels that drain directly to the pituitary gland. These ns-neurons are innervated and regulated by neurons from other brain regions. The anterior portion of the pituitary or adenohypophysis consists of several cell types that produce tropic hormones responsible for regulating the other endocrine glands as well as some nonendocrine targets. Once the releasing hormones reach the adenohypophysis, each stimulates (or, in some cases, inhibits) the release of a specific tropic hormone. In the case of the adrenal cortex, the hypothalamus secretes corticotropin-releasing hormone (CRH, or corticotropin-releasing factor, CRF) that stimulates one adenohypophysial cell type (the corticotrope) to release corticotropin (ACTH) into the general circulation. ACTH, in turn, stimulates certain cells in the adrenal cortex to synthesize and release steroid hormones called glucocorticoids (cortisol and/or corticosterone) into the blood. The glucocorticoids stimulate protein catabolism in muscle and the conversion of amino acids and fatty acids to glucose (gluconeogenesis) by the liver. These steroids also inhibit glucose uptake by cells other than those of the nervous system, resulting in a marked elevation of blood glucose that is then utilized by the brain. Together, these three anatomical structures comprise the hypothalamus-pituitary-adrenocortical (HPA) axis. Corticosteroids feedback primarily at the brain to repress the release of CRH, thereby reducing ACTH secretion and, ultimately, cortisol secretion (Allison and Omeljaniuk, 1998).

All of the components of the mammalian HPA axis are present in fishes (Fig. 22.1). CRH-like neurons have been described in the preoptic area and the nucleus lateralis tuberis (NLT) of chinook salmon, *Oncorhynchus tshawytscha* (Matz and Hofeldt, 1999), goldfish, *Carassius auratus*, and white sucker, *Catostomus commersoni* (Lederis et al., 1994). CRH immunoreactivity was also reported in the comparable brain regions of the sturgeon, *Acipenser ruthenus* (Gonzalez et al., 1992). These CRH-like molecules in fishes are very similar to mammalian CRH (see Lovejoy and Balment, 1999), and we will refer to the piscine form simply as CRH. Elevated CRH levels in the preoptic area of subordinate rainbow trout, *Oncorhynchus mykiss* (Doyon et al., 2003) are consistent with observations of elevated cortisol in subordinate arctic charr (Øverli et al., 1999).

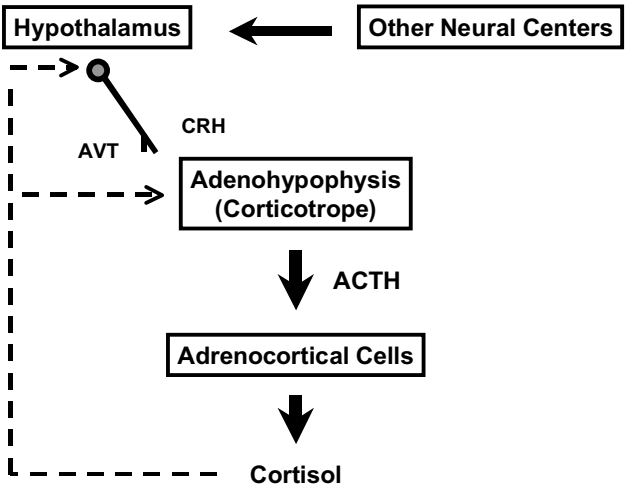

Fig. 22.1 The hypothalamus-pituitary-adrenocortical (HPA) axis in gnathostome fishes. This scheme is based on teleosts, but the axis of elasmobranchs is similar except that the principal steroid synthesized by the adrenocortical cells is 1α-hydroxycorticosterone and in lungfishes it is corticosterone or deoxycorticosterone The dotted line represents negative feedback by cortisol. See text for explanation.

In white sucker (Yulis and Lederis, 1987) and rainbow trout (Ando et al., 1999), CRH is colocalized with the peptide arginine vasotocin (AVT) in magnocellular cells of the preoptic nucleus (NPO). CRH peptide also is found in the white sucker NLT together with urotensin-I, a CRH-like peptide chemically related to mammalian urocortin (see Lovejoy and Balment, 1999) and also expressed in the hypothalamus of goldfish (Bernier et al., 1999) and rainbow trout (Barsyte et al., 1999). Lesion of the NPO in goldfish abolished circulating cortisol with no affect on ACTH levels, whereas NLT lesions reduced the pituitary content of ACTH as well as circulating levels of cortisol, possibly indicating different physiological roles for these two regions with respect to CRH (Lederis et al., 1994). Both arginine vasopressin (AVP), a molecule closely related to AVT, and urocortin may play a role in the HPA axis response to stress in mammals; however, the roles of AVT and CRH-like peptides have not been extensively studied in teleosts. In mammals, AVP colocalizes with CRH and synergistically enhances corticotrope response to CRH. In fishes, AVT potentiates CRH-induced ACTH release in rainbow trout (Baker et al., 1996), but not in goldfish (Fryer et al., 1995).

Corticotropes are present in the fish adenohypophysis (*pars distalis* or *rostral pars distalis*) of the pituitary and secrete ACTH (see Holmes and Ball, 1974). A vascular portal system conducts CRH-like peptides to the pituitary in elasmobranchs and other bony fish groups as in tetrapods. Teleosts lack a portal system, and the corticotropes are innervated by neurons that originate in the hypothalamus of the brain and secrete CRH (Olivereau et al., 1984; Lederis et al., 1994; Matz and Hofeldt, 1999). When the hypothalamic CRH neurons are stimulated, they release CRH that causes a release of ACTH that in, turn, travels through the general circulation and stimulates corticosteroid secretion by the adrenocortical cells (see Lederis et al., 1994). Thyrotropes of salmonids also are innervated by CRH neurons (Matz and Hofeldt, 1999), and CRH can release TSH in teleosts (Larsen et al., 1998).

Release of CRH from hypothalamic neurons ending in the rainbow trout pituitary is caused by serotonin (5-HT) acting on 5-HT_{1A} receptors on the cell membrane of CRH neurons (Winberg et al., 1997). 5-HT also peaks in the brain of coho salmon (*Oncorhynchus kisutch*) during smoltification, which may be associated with the rise in cortisol secretion at this time (Specker, 1982; Dickhoff et al., 1990). Thus, regulation of the HPA axis by some environmental stimuli may operate through 5-HT neurons.

The interrenal gland or adrenocortical tissue of fishes is analogous to the adrenal cortex of mammals but differs somewhat from mammals both anatomically and physiologically. Numerous in-depth reviews of the adrenocortical anatomy of fishes are available (Chester Jones et al., 1959; Nandi, 1962; Idler and Truscott, 1972; Jorgenson, 1976; Matsumoto and Ishii, 1987; Norris, 1997; Bentley, 1998). The adrenocortical tissue is located between the kidneys in elasmobranchs (truly interrenal; Matsumoto and Ishii, 1987), but is embedded as 'yellow corpuscles' in the more anterior portion of the kidney of holostean fishes (de Smet, 1962; Youson and Butler, 1976) and chondrostean fishes (Bhattacharyya et al., 1981). In teleostean fishes, the adrenocortical tissue appears in the form of scattered clumps of cells distributed within the lymphoid head kidney (Nandi, 1962; Matsumoto and Ishii, 1987). Fishes also have cells that are homologous to the mammalian adrenal medulla. These cells are referred to as chromaffin cells because of their affinity for staining with certain chromium-based dyes (see Norris, 1997). Fish chromaffin cells secrete catecholamines, epinephrine and norepinephrine, just like cells of the

mammalian adrenal medulla. Chromaffin cells are associated with the anterior kidney vasculature in fishes but also may be embedded within the kidney. There is no 'cortex' and 'medulla' relationship as in mammals. Because of its functional similarity to the mammal adrenal cortex (see Norris, 1997), the interrenal tissue of fishes will be referred to as adrenocortical tissue here. Although often called the HPI (hypothalamus-pituitary-interrenal) axis in fishes, this system will be called the HPA axis in this chapter.

The adrenocortical tissue of fishes typically secretes a single corticosteroid. Teleosts and most other bony fishes secrete primarily cortisol, whereas elasmobranchs (e.g., sharks) secrete a unique corticosteroid, 1α-hydroxycorticosterone. Lungfishes, like their tetrapod relatives, primarily secrete corticosterone or deoxycorticosterone. Only traces of aldosterone, the mineralocorticoid secreted primarily by adrenocortical tissue of tetrapods, have been described in fishes. Cortisol in teleosts apparently functions as both a mineralocorticoid (influences ion balance like aldosterone in tetrapods) and as a glucocorticoid (regulates certain aspects of carbohydrate metabolism). Relatively little information is available on the actions of corticosteroids in fishes other than teleosts and, hence, we will emphasize teleosts in the following discussions. Functional studies of the HPA axis and corticosteroids in other groups of fishes are sorely needed.

Diel and seasonal rhythms for plasma corticosteroid levels have been described in a number of teleosts including rainbow trout (e.g., Bry, 1982), brown trout, *Salmo trutta* (Pickering and Pottinger, 1983), and banded killifish, *Fundulus diaphanous* (Fivizzani et al., 1984), although juvenile fishes usually lack distinct rhythms (e.g., Strange et al., 1977). Peak cortisol levels occur during the night (scotophase peak) in these species, and a seasonal shift in the timing of this peak has been described for brown trout (Pickering and Pottinger, 1983). Cortisol surges have also been reported following feeding (Bry, 1982; Pickering and Pottinger, 1983), but this fact is not often addressed in laboratory studies.

PLASMA-BINDING PROTEINS

In mammals, most of the cortisol in the blood is bound to a protein. This binding protein is known as corticosteroid-binding globulin (CBG) or transcortin. Protein-bound cortisol is in equilibrium with free cortisol found in the plasma in minute traces. The present consensus is that only

free corticosteroid is available to bind to membrane receptors on the surface of target cells or, more typically, to enter target cells and bind to cytosolic receptors. The CBG-bound corticosteroid represents a ready reservoir to replace free cortisol that enters the target cells or is metabolized and excreted (see Norris, 1997). Apparently, there are plasma proteins in all vertebrates that are capable of binding cortisol, although the percentage of bound cortisol is much lower in fishes than in mammals, as indicated by early studies (e.g., Seal and Doe, 1965; Idler and Freeman, 1968). As in mammals, there appears to be one protein that acts like a CBG with a high affinity but low capacity for cortisol. Serum albumin has a high capacity for corticosteroids but very low affinity and is not a major corticosteroid binding protein in mammals. In fishes, however, Caldwell et al., (1991) reported that about 30-40% of the circulating cortisol in immature and mature male rainbow trout was weakly bound to serum albumin and about 44% was free, with less than 20% bound to a CBG-like protein. Almost half of the plasma cortisol was bound to CBG in mature females with only about 22% free. The differences in these data have not been explained and are in contrast to spawning female sockeye salmon (*Oncorhynchus nerka*) that appear to have very low protein binding (Idler and Freeman, 1968) and very high levels of cortisol (Idler and Freeman, 1968; Kubokawa et al., 2001). Some careful studies of cortisol binding in fishes of all types are necessary to understand the role of these binding proteins.

CORTICOSTEROID RECEPTORS (CR) IN FISHES

GR/MR Structure

Corticosteroids exert their actions at target tissues through binding intracellular receptors that are members of the steroid/thyroid hormone and retinoic acid superfamily of receptors. Although plasma membrane-bound receptors have been described in mammals and amphibians, the appropriate studies have not been done in fishes to indicate their presence. In mammals, corticosteroids bind two types of receptors called mineralocorticoid (MR) and glucocorticoid (GR) receptors (see Jenkins et al., 2001). These receptors, also called GR I (MR) and GR II (GR), are often referred to as 'nuclear receptors' because they function within the nucleus as transcription factors when activated by binding of ligands (e.g., cortisol). The occupied receptor (ligand plus receptor) then binds to

specific sites on DNA called hormone-response elements (HREs). The overall structure of these receptors consists of highly conserved modular domains that perform specific functions. Beginning at the amino-terminus is a variable A/B domain that mediates ligand-independent transcriptional regulation through interactions with transcriptional regulators. After the A/B domain is a conserved C domain that contains the DNA-binding domain (DBD), followed by a D domain that mediates receptor conformational changes, and lastly a conserved E domain possessing the ligand-binding domain (LBD).

Three distinct cortisol receptors are identified in rainbow trout (rt): rt GR 1, rt GR 2 and MR (Colombe et al., 2000; Bury et al., 2003). The discovery of an MR in fish is particularly interesting considering that most fish, including rainbow trout, fail to produce significant amounts of aldosterone. A unique structural feature of fish GR compared to tetrapod GR is the presence of an extra 4 and 9 amino acids between the zinc fingers of the DBD of rt GR 1 and rt GR 2, respectively (Bury et al., 2003). Zinc fingers are present in all steroid receptors and are normally highly conserved. These domains are composed of amino acid loop domains that complex Zn^{2+} and aid binding of the occupied receptor to HREs. How these additional amino acids affect the affinity for HREs or the manner in which they affect transcriptional regulation is currently unexplored. Mammalian MR and GR, the latter of which has GRa and GRb splice variants (Oakley et al., 1996), arose from a gene duplication event that also occurred in fishes, yielding fish MR and GR. rt GR 1 and GR 2, however, arose from a separate gene duplication event in GR that did not occur in tetrapods. Not surprisingly, both rt GR1 and rt GR2 are more similar to mammalian GR II, than they are even to fish MR. To make matters even more confusing, 4 distinct corticosteroid receptors recently have been sequenced in the cichlid fish, *Haplochromis burtoni* (Greenwood et al., 2003). These have been named hbMR, hbGR1, hbGR2a and hbGR2b, with the latter two being splice variants of the same gene. A GR has also been identified for halibut (*Paralichthys olivaceus;* = Japanese flounder) (Tokuda, 1998). Phylogenetic relationships of fish and human corticosteroid receptors sequenced to date are shown in Fig. 22.2. To summarize, two gene duplication events occurred in fishes, the first ultimately giving rise to MR and GR (prior to the divergence of tetrapods from fishes), and the second giving rise to rt GR1 and rt GR2 or their homologues in other fishes (e.g., hb GR 2 and hb GR1).

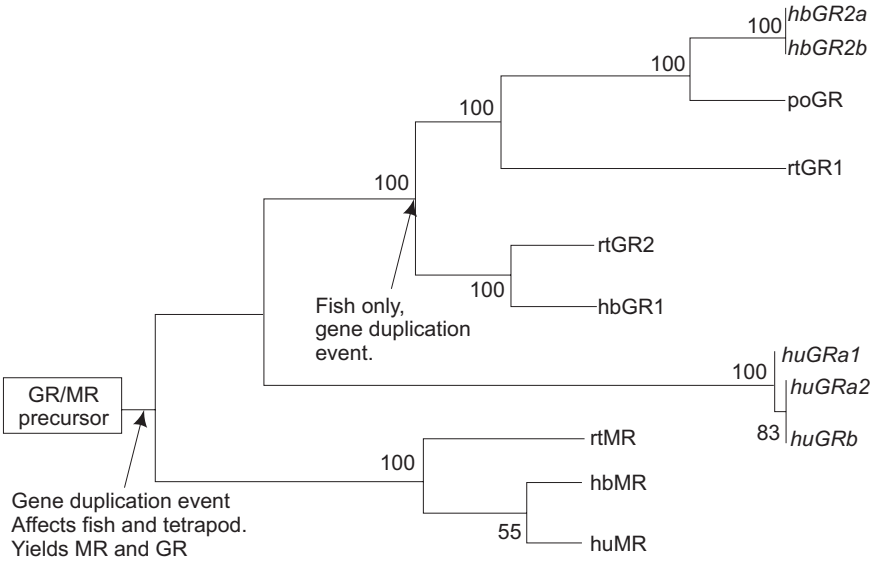

Fig. 22.2 Phylogenetic tree of all fish and human CR genes and splice variants sequenced to date. Numbers at branches indicate bootstrap values. Labels in italics and on the same branch are splice variants of the same gene. Hb = *Haplochromis burtoni* (a cichlid); po = *Paralichthys olivaceus* (bastard halibut); rt = *Oncorhynchus mykiss* (rainbow trout); hu = human.

GR/MR Activation

Whereas other steroid receptors are distributed throughout the cytoplasm and the nucleus when inactive, the unbound and inactive receptors for corticosteroids are largely retained in the cytoplasm. Prior to ligand binding, mammalian corticosteroid receptors are maintained in an inactive but high cortisol-affinity state through stabilizing interactions with heat shock proteins (hsp) 40, 70, and 90 and two other proteins, p23 and p60 (also called HOP) (Dittmar et al., 1998; Kosano et al., 1998). The formation of a high-affinity state is actually an ATP-dependent-two-stage process. The first ATP-dependent stage appears to be a priming reaction, wherein hsp40 facilitates hsp70 binding to the receptor. The second ATP-dependent stage appears to be a receptor-activating step, where p23 and HOP facilitate the binding of receptor to hsp90, which stabilizes the receptor in a conformation that exposes the hydrophobic ligand-binding domain (Kanelakis et al., 2002). Upon ligand binding, GR undergoes a conformational change, disassociates with the heat shock proteins and translocates to the nucleus where it can modify expression of multiple

genes through a variety of mechanisms. The nuclear GR can homodimerize and bind to a glucocorticoid response element (GRE), which can cause either transcriptional activation or repression of target genes. The translocated steroid receptor complex can also associate with coactivators that confer histone deacetylase activity. Deacetylation of histones weakens the histone-DNA interaction that would otherwise occlude transcriptional machinery from accessing promoter regions on genes. Activated GR also can interact with other transcriptional regulators such as AP-1 to regulate genes that lack a GRE.

GR and MR in Fishes

Traditionally, fishes were believed to lack a mineralocorticoid receptor, partly because one simply hadn't been found, and partly because most fishes lack significant levels of aldosterone, the primary mineralocorticoid in mammals. Additionally, the gills and intestines of fishes contain glucocorticoid receptors that when activated by cortisol, can regulate at least some of the mineralocorticoid-like functions these tissues impart, i.e. adaptation to sea/fresh water. Hence, the evidence suggests that cortisol acts through glucocorticoid receptors to perform both glucocorticoid functions and mineralocorticoid functions, the latter being relegated in tetrapods to aldosterone and MRs. Another difference is revealed by sequence analysis of the various fish GR's, which suggests that fish GR is not the precursor to tetrapod MR, but that both are derived from some common earlier gene (Ducouret et al., 1995).

Not long after rainbow trout GR was sequenced, an MR was cloned and sequenced in rainbow trout (Colombe et al., 2000) and evidence has already been presented for its mineralocorticoid functional activity in branchial chloride cells (Sloman et al., 2001a). rt MR shares 69% homology with human MR and only 35% homology with rt GR 1. rt MR, like its mammalian counterpart, has a higher affinity for cortisol than for aldosterone in ligand binding studies (Colombe et al., 2000). Unfortunately, knowledge of fish MR distribution, regulation, and activity is lacking. Furthermore, the discovery of fish MR is unlikely to warrant the revoking of all mineralocorticoid functions previously attributed to GR. Studies have shown that GR mRNA expression in gill branchial chloride cells of chum salmon fry (*Oncorhynchus keta*) is colocalized in filamentous chloride cells, along with the GR protein (Uchida et al., 1998). The GR protein is identified readily using antibodies unlikely to cross-react with

the newly discovered MR protein due to the absence of epitope homology. This fish GR was also upregulated following seawater adaptation. However, other studies in rainbow trout suggest that MR has gill mineralocorticoid functions (Sloman et al., 2001a). In these studies, rainbow trout exposed to artificial soft water (ion-deficient freshwater) experienced proliferation of branchial chloride cells that was inhibited by treatment with an MR-specific inhibitor, spironolactone, but not with a GR-specific inhibitor, RU-486.

Recently, the picture became even more complex with the discovery of rt GR2, sequenced from intestinal cDNA (Bury et al., 2003). Here, rt GR2 most likely arose from gene duplication early in the teleost lineage. Currently, no other studies on rt GR2 have been published and, therefore, nothing is known about it's tissue distribution (other than it is transcribed in rainbow trout intestine) or its functional significance. However, rt GR 2 appears to have a higher affinity for cortisol compared to rt GR1 based on radioligand studies in transfected cells (Bury et al., 2003). The presence of three receptors with different affinities for cortisol raises the possibility that a tissue's response to cortisol may change with increasing levels of cortisol as receptors with lower affinities are bound and activated at higher cortisol levels, a phenomenon that certainly occurs in mammals. A possible example of this in tilapia (*Oreochromis mossambicus*) gill is demonstrated by the finding that low doses of cortisol (0.28uM) protect against C^{2+} induced necrosis of chloride cells, but higher doses (0.83uM) induce apoptosis (Bury et al., 1998). Currently though, cortisol's affinity for rt MR relative to GR 1 and GR 2 is unknown, and studies have yet to conclusively identify co-expression of multiple receptor types in any fish tissue.

GR/MR Interactions with Heatshock Proteins in Fishes

Heatshock proteins (hsps), named after their adaptive response to heat shock, comprise several classes of proteins that all seem to support the function and stability of other proteins in some way. Hsps promote proper folding of nascent polypeptides, correct improperly folded proteins and partially denatured proteins, prevent protein aggregation (Fink, 1999) and facilitate either repair or degradation of improperly folded proteins (Kiang and Tsokos, 1998). Hsps are critical requirements for corticosteroid-GR binding and have recently received considerable attention in fishes (Basu et al., 2002; Murtha and Keller, 2003). In addition to mediating a tissue

response to some form of stress, such as heat shock or ether treatment, regulation of hsps appears to be a mechanism by which the tissue response to corticosteroids is tailored. Both hsp induction and CR signaling are responses to stress, and increasing evidence suggests that regulation of their functions is intimately connected and, in some cases, possibly reciprocally regulated. Hsps have cell survival functions in addition to direct interactions with corticosteroid receptors, and they are found in bacteria which lack corticosteroid receptors altogether (Fink, 1999). Heat shock as a cellular stressor has been well studied in fish *in vivo* (Basu et al., 2002; Murtha and Keller, 2003) and hsps have been studied extensively in organisms ranging from bacteria to mammals (Fink, 1999). The importance of hsps in preventing aggregation of proteins with exposed hydrophobic regions is underscored by increasing evidence implicating protein aggregation in multiple aging diseases, such as Alzheimer's disease and Parkinson's disease. As mentioned above, hsp 40, 70, and 90, along with two other proteins p23 and HOP, are also absolute requirements for steroid receptor function, and are sufficient for reconstitution of CR activity *in vitro* (Dittmar et al., 1998). Considering that CRs interact with hydrophobic corticosteroid molecules, hsps possibly serve to keep CR in the proper ligand-ready conformation while simultaneously preventing aggregation with other CR molecules, or other proteins with hydrophobic residues.

In most fish studies, cortisol administered prior to heat shock actually attenuates the heat-induced expression of hsps. For example, pre-treating rainbow trout primary hepatocytes with cortisol significantly attenuated the post-heat shock hsp70 induction in a dose-dependent manner (Boone and Vijayan, 2002a). Handling stress also attenuated hsp70 and hsp30 post-heat shock induction in gill of cutthroat trout (Ackerman et al., 2000). While the hsp response serves the immediate needs of a cell in protecting against protein aggregation or against proteotoxicity, CR signaling is part of an evolved organismal response to a real or perceived threat to survival. The goal of the former is to protect a specific tissue, whereas the latter is to ensure survival of the organism. To some extent, the HPA response to stress and a tissue's hsp response to stress may serve antagonistic purposes. In support of this, heat-shocked trout hepatocytes exhibit elevated hsp70 levels, reduced GR levels, and reduced glucocorticoid-induced glucose release (Boone and Vijayan, 2002a; Boone et al., 2002; Vijayan et al., 2003). Such a response *in vivo* could severely impair the hepatic metabolic adjustments necessary to survive a major

stress. Conversely, several studies show that cortisol or handling stress administered prior to heat shock actually attenuates the heat shock-induced elevation of hsps. For example, cortisol attenuates the post-heat shock hsp induction in gill, liver, hepatocytes, and brain (Ackerman et al., 2000; Basu et al., 2001, Sathiyaa et al., 2001; Boone and Vijayan, 2002b). The attenuated hsp70 response appears to be a direct consequence of the cortisol-induced down regulation of GR. Inhibiting GR breakdown restores the hsp70 induction in cortisol-treated, heat-shocked trout hepatocytes (Boone and Vijayan, 2002a).

The interaction of hsps and the HPA signaling pathway is surely more complex than a simple counter regulatory model would predict. Recent *in vivo* studies have shed new light on this picture. Vijayan et al. (2003) actually show that both cortisol and handling stress increase production of the constitutive hsps, hsp70 and hsp90, in rainbow trout liver. With higher levels of these constitutive heat shock proteins, perhaps a dramatic hsp70 induction is not necessary to cope with cellular stress, and hence the hsp70 response to heat shock is attenuated. Attenuating the hsp70 response to heat shock also may prevent the decrease in GR signaling reported at elevated hsp70 levels (Boone and Vijayan, 2002a). Furthermore, elevating hsp90, which binds to and holds the ligand-free GR in a high-affinity state, will likely increase GR sensitivity, possibly offsetting the GR downregulation caused by elevated cortisol. The fact that GR signaling was adequately maintained is supported by the finding that stressed and cortisol-treated trout still had elevated blood glucose and liver glycogen content and increased mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK), a major liver gluconeogenic enzyme, despite having decreased liver GR levels (Boone et al., 2002). Perhaps the most parsimonious explanation is that signaling through cortisol receptors increases liver resistance to baseline levels of cellular stress and elevates the cellular threshold for hsp induction while simultaneously affecting the HPA-driven, gluconeogenic response. Certainly more studies, and especially *in vivo* studies, are required to validate these new findings and bring the increasingly complex interactions of fish hsps and CRs into focus.

GR/MR Tissue Distribution and Regulation in Fishes

In fishes, corticosteroid receptors are expressed in a variety of tissues including liver, gill, muscle, kidney, blood, and brain. The promiscuous

nature of corticosteroids has earned them the nickname 'dirty hormones' because they truly seem to go everywhere and do everything. Some tissues, such as gill, appear to regulate their response to corticosteroids through altered expression and function of corticosteroid receptors, such as during seasonal changes and under different environmental or physiological conditions. Understanding the various tissue responses to cortisol will be aided by knowledge of how the various receptor types are distributed and regulated. To this end, radioligand studies, in situ hybridization studies, and immunohistochemical approaches have all been used to identify and characterize cortisol receptors in the tissues of fishes. To minimize ambiguity, GR will be used to indicate a receptor that could be any GR receptor, i.e., rt GR 1 or rt GR 2, but not MR. CR (corticosteroid receptors) will indicate a receptor for cortisol identified by ligand-binding studies, which could be any splice variant or combination of MRs and GRs.

CORTISOL AND PHYSIOLOGICAL FUNCTIONS

Cortisol and Ionic-Osmotic Regulation

Ionic and osmotic regulation in fishes living in either seawater (SW) or freshwater (FW) is accomplished by the cooperative actions of gill, intestine, kidney and sometimes the urinary bladder. The functions of these organs are influenced by a variety of hormones, including prolactin, growth hormone (GH), thyroid hormones, catecholamines, and cortisol. Catecholamines, which appear to be involved in the early stress response (see below), can cause acute losses of Na^+ and Cl^- (see McDonald and Milligan, 1997). Elevation in plasma cortisol is observed in SW-adapted eels following transfer to FW, and this persists for several days (Hirano, 1969). Plasma cortisol also is elevated in salmonids during the parr-smolt transformation prior to seaward migration (Hoar, 1988; Avella et al., 1990; Dickhoff et al., 1990).

Plasma cortisol levels are elevated following exposure to a great variety of stressors, and fatalities following acute, severe stress usually are accompanied by ion losses. These stressors do not affect chloride cell numbers in the gills of rainbow trout, (Sloman et al., 2000, 2001b), suggesting they alter the activity of existing chloride cells. However, cortisol treatment had no effect on plasma ion levels or Na^+/K^+-ATPase in gills of cutthroat trout, *Oncorhynchus clarki* (Morgan and Iwama, 1996).

Changes in gill structure and function under osmotic stress appear to be induced by thyroid hormones (see McDonald and Milligan, 1997; Kelly and Wood, 2001), although cortisol may augment the actions of thyroid hormones (Kelly and Wood, 2001). However, in hypophysectomized juvenile coho salmon, cortisol or GH treatment increased both chloride cell number and Na^+/K^+-ATPase activity in the gills (Björnsson et al., 1987; Richman et al., 1987b). Obviously, careful experiments are needed to ascertain the precise role of cortisol in ionic and osmotic balance related to developmental stage. Most work has been done on the role of cortisol in FW fishes or euryhaline fishes undergoing physiological adaptation to FW or SW (see discussion of smoltification in salmonids below) and little is known of its involvement after adaptation when levels of cortisol return to FW levels. Studies that show no elevation of plasma cortisol concentrations when the general functional level of the entire HPA axis is elevated (see Norris, 2000) illustrate the danger of assuming too much from one static measurement of HPA function.

Cortisol signaling through CRs is believed to mediate gill physiological changes by regulating chloride cell expression of the Na^+/K^+-ATPase and by preparing chloride cells for ion exchange in either SW or FW. The Na^+/K^+-ATPase is an ion transporter found in the basolateral membrane of chloride cells and is necessary for acclimation to both FW and SW. Changes in the sensitivity and abundance of gill CRs occur with stress and cortisol administration, and are thought to help mediate gill physiological responses to osmotic challenges, such as transitions between FW and SW. Repeated handling stress in juvenile rainbow trout elevates cortisol, but reduces both CR numbers and the cortisol-mediated induction of Na^+/K^+-ATPase (Shrimpton and McCormick, 1999). In coho salmon, both stress and cortisol treatment decreased gill responsiveness to cortisol by reducing CR abundance, with stress also reducing CR sensitivity (Shrimpton and Randall, 1994). The significance of these responses is strengthened by the finding that 14 days of cortisol treatment in juvenile Atlantic salmon (*Salmo salar*) increases gill Na^+/K^+-ATPase activity and increases salinity tolerance, indicated by lower plasma Na+ levels when transferred to SW (McCormick, 1996). These findings suggest that activation of the HPA axis can enhance SW tolerance without actual exposure to SW.

Although cortisol elevation is associated with gill histological and osmoregulatory changes during both the transition from FW to SW (smoltification) and during the return to FW in spawning salmon, studies suggest that at least some fish can acclimate to osmotic challenge without

significant cortisol elevation. Instead, regulation of gill CR abundance and sensitivity occurs after the transfer from FW to SW. For example, at 1 and 4 days post transfer from FW to SW, the number of gill CRs is increased (increased B_{max}) in the euryhaline tilapia (Dean et al., 2003). These changes correlated with increased salinity tolerance and occurred without a significant increase in cortisol, despite frequent sampling. Similarly, in chum salmon fry transferred to SW for 3 weeks, no significant change in cortisol levels was observed. However, GR transcript levels increased significantly in filamentous chloride cells coincident with increased gill Na^+/K^+-ATPase activity, and lamellar chloride cells bearing GR transcripts disappeared entirely (Uchida et al., 1998). Filamentous and lamellar chloride cells are named for their anatomical location in the gill arch and display different osmoregulatory properties when exposed to SW or FW. GRs also were found in undifferentiated cells, suggesting that signaling through GR may help regulate differentiation of filamentous and lamellar chloride cells. Perhaps upregulating gill CRs is an evolved mechanism for acquiring salinity tolerance that obviates an HPA-mediated organismal stress response and the deleterious side effects, such as immunosuppresion, that often accompany HPA activation.

The ability of fishes to tolerate salinity changes is certainly influenced by more than just cortisol signaling through CRs. Furthermore, gill osmoregulatory capabilities also can be enhanced prior to a transition between FW and SW and during seasonal changes, rather than just as a response to stress, cortisol or osmotic challenges. Several hormones involved in smoltification or FW migration, including GH, prolactin and triiodothyronine (T_3), are known to modulate gill responsiveness to CR and to promote either SW or FW tolerance. These same hormones may be involved in seasonal increases in CR abundance and affinity and gill Na^+/K^+-ATPase activity as these are all increased in Atlantic salmon that fail to smolt or experience a dramatic springtime cortisol elevation during their first year (Shrimpton and McCormick, 1998a). During smoltification, GH, T_3, and cortisol are elevated and studies on Atlantic Salmon have demonstrated that GH and T_3 synergize to increase gill CR levels while GH increases CR affinity for cortisol and increases Na^+/K^+-ATPase activity (Shrimpton and McCormick, 1998b). GH also synergizes with cortisol administration to increase Na^+/K^+-ATPase activity and salinity tolerance (McCormick, 1996). Prolactin, which is associated with FW acclimation in spawning salmon, did not upregulate cytosolic CR or increase Na^+/K^+-ATPase activity, but did reduce some of the effects of

GH (Shrimpton and McCormick, 1998b). The change in CR affinity induced by GH is particularly interesting because one possible explanation is that a change occurred in the type of corticosteroid receptor expressed or activated. Another possibility, however, is that changes in CR affinity are mediated by interactions with hsps, as discussed by Shrimpton and McCormick (1998a). Future studies should address whether acclimation to SW and FW and the corresponding alternate differentiation of chloride cells are mediated by: (1) preferential binding to different types or combinations of corticosteroid receptors; (2) the effects of other hormones, such as GH, prolactin, and T_3 or; (3) altered CR interactions with hsps.

CORTISOL AND METABOLISM

Glucocorticoids were so named for their ability to stimulate the formation of glucose through the process of gluconeogenesis from amino acids and fatty acids. In mammals, this action is mediated through PEPCK in liver (Gunn et al., 1975; Sharma and Patnaik, 1983), the rate-limiting enzyme for gluconeogenesis (Huibregtse et al., 1976). Glucocorticoids also stimulate protein breakdown in muscles to provide amino acids for gluconeogenesis in the liver (see Norris, 1997). In rainbow trout, cortisol affects the levels of key gluconeogenic enzymes (Freeman and Idler, 1973), suggesting a similar gluconeogenic role for cortisol in fish. Cortisol treatment and stress elicit hyperglycemia in a wide variety of species (see Pankhurst and van der Kraak, 1997; Vijayan et al., 1997). Foster and Moon (1986) observed increased PEPCK activity following cortisol treatment of American eels, *Anguilla rostrata*, supporting a gluconeogenic role for cortisol. However plasma levels of glucose were unaffected by chronic cortisol administration to rainbow trout (i.e., no gluconeogenic effect) but plasma amino acid levels were elevated (Andersen et al., 1991), supporting at least a protein catabolic action for cortisol in this species.

Exercise of fishes to exhaustion results in a marked depletion of muscle glycogen (see Kieffer, 2000) as well as a marked increase in plasma cortisol levels (Pagnotta et al., 1994; Milligan, 1996, 2003). High cortisol prevents or attenuates replenishment of glycogen stores (glyconeogenesis) following exhaustive exercise (see Milligan, 2003). Placing rainbow trout in low-velocity flowing water after exhaustive exercise reduced plasma cortisol levels and accelerated glycogen resynthesis when compared with

trout recovering in still waters (Milligan et al., 2000). Treatment of rainbow trout with metyrapone, a drug that prevents cortisol synthesis, prior to exercise accelerated the rate of restoration of muscle glycogen stores compared to controls (Milligan, 2003). Inactivation of phosphorylase, the key glycogen-degrading enzyme, and activation of glycogen synthase, the enzyme responsible for glycogen resynthesis, were accelerated in metyrapone-treated fish. Furthermore, cortisol injections prevented glycogen resynthesis.

The metabolic role for cortisol in fishes is also supported by the observation that feeding can cause increased secretion of cortisol (Bry, 1982; Pickering and Pottinger, 1983). Elevated cortisol following a meal may help convert absorbed amino acids into carbohydrate and then to lipid for storage. Conversely, food deprivation in rainbow trout also stimulates cortisol synthesis, further evidence for a protein catabolic and possibly gluconeogenic role for cortisol (Blom et al., 2000).

THE STRESS RESPONSE IN FISHES

Everyone seems to understand the concept of stress, yet it continues to be an elusive term when one tries to provide a concise definition (see Levine and Ursin, 1991; Toates, 1995). Because of the psychological side of stress, it is difficult to define it in wild or laboratory animals where it is difficult to explore the psychological side of stress. Although authorities cannot agree on a precise definition, Chrousos (1997) describes stress as any threat to homeostasis. A stressor is then defined as anything that brings about a physiological stress response in the HPA axis. The stress response, i.e., the physiological changes that occur following exposure to a stressor, is easier to define because one can describe the physiological parameters involved including an initial elevation of plasma catecholamine (epinephrine and norepinephrine) levels followed soon by increased plasma levels of ACTH and cortisol.

Physiological changes of the vertebrate stress response were first described for mammals by Hans Selye in his *General Adaptation Syndrome* (see Selye, 1971, 1973), The stress response involved an initial alarm reaction mediated by a transient secretion of epinephrine and/or norepinephrine from the chromaffin cells and a sustained increase in cortisol or corticosterone if the stressor remained (called the resistance phase by Selye). The alarm reaction of Selye is similar to Cannon's 'emergency response' except for the elevation of glucocorticoids in the

former. The stress response is, thus, an adaptive response operating through the chromaffin cells and the HPA axis that directs metabolism through glycogenolysis, lipolysis, and gluconeogenesis to provide increased glucose for neural activity. Removal of the stressor results in a rapid return of plasma cortisol to normal, usually within 24 h. If the stressor is not removed or successfully mediated, glucocorticoid secretion remains elevated to aid the animal metabolically in adapting to the continued presence of the stressor. However, prolonged exposure to the stressor will result in exhaustion of the animal and decreased immunocompetence, and can also lead to death. Stressed animals can be characterized as exhibiting decreased feeding, weight loss, cessation of reproductive activity, and increased incidence of disease (Toates, 1995).

The primary stress response of fishes involves both catecholamines from the chromaffin cells and cortisol from the adrenocortical cells (see Barton, 1997, 2002; Sumpter, 1997). However, it is exceedingly difficult to study the role of the chromaffin cells in the stress response in natural populations of fishes because the rapid response to stress makes establishing a resting level of plasma catecholamines virtually impossible unless the fish has been prepared with an indwelling cannula so that a sample can be obtained without disturbing the fish (see McDonald and Milligan, 1997). For example, large cannulated trout have a resting epinephrine level of 1-10nM, whereas rainbow trout sampled from the caudal vein within 30 seconds of capture have a plasma level of 100 nM. Consequently, investigators usually rely on the slower and more prolonged secretion of cortisol as an indicator of acute stress (see Table 22.1).

In addition to the primary stress response, secondary responses (changes in blood and other tissues), and tertiary responses (individual and population effects) to stress can occur in fishes (see, Wedemeyer et al., 1990; Barton and Iwama, 1991; Sumpter, 1997; Barton, 2002). Secondary responses are the consequence of stress hormone actions on metabolic and ionic processes that can bring about altered tertiary responses such as lowered immunocompetence, abnormal behavior, and reduced reproductive success.

Mobilization of the HPA axis following even minor disturbances of fishes also can be a rapid event, especially in salmonids where plasma cortisol can be elevated significantly within 10 to 15 minutes following exposure to a stressor (see Barton and Iwama, 1991) even at very low water temperatures (Norris et al., 1997). Stressors of fishes include subtle activities such as netting a fish and immediately releasing it, handling a

Table 22.1 Levels of cortisol[1] (ng/mL) in unstressed and stressed fishes reported after publication of the extensive summary by Barton and Iwama (1991).

Species[2]	*Unstressed*	*Stressed*	*Time & Type*[3]	*Increase*	*Reference*[4]
Australian lungfish, *Neoceratodus forsteri* (j)	ND	ND	C, p	–	(11)
Pallid sturgeon, *Scaphirhynchus albus* (j)	2.3 ± 0.3	3.0 ± 0.3	A, m	–	(2)
White sturgeon, *Acipenser transmontanus*					
(a,m; cannulated)	8.6	≈40	A, m	4.7X	(3)
(a,m; cannulated)	≈5	≈33	A, p	6.6X	(3)
Paddlefish, *Polyodon spathula* (j)	2.2 ± 0.06	11 ± 1.8	A, m	5X	(2)
Florida gar, *Lepisosteus platyrhincus* (a)	≈2	≈9	C, p	4.5X	(14)
Brown trout, *Salmo trutta* (j)	1.0 ± 0.3	94 ± 11	A, m	9X	(2)
(a)	≈20	≈80	A, n	4X	(9)
(j + a)	≈10	≈225	C, p	22.5X	(12)
(j)	1.3±0.6	≈160	A, p	123X	(21)
Atlantic salmon, *Salmo salar* (j)	≈5	≈53	A, p	10.6X	(22a)
?	≈10	≈50	A, r	5X	(25)
Rainbow trout, *Oncorhynchus mykiss* (a)	≈19-34	≈70-85	A, n	2.5-3.7X	(13)
(a; monthly sampling)	NA	35-110	A, p	–	(18)
(j)	1.7 ± 0.05	43 ±3.5	A, m	25.3X	(2)
(j)	0.7±0.1	≈36	A, p	51X	(21)
(a)	–	49-209	C, p	–	(5)
(a; cannulated)	≈75	≈160	C, r	2.1X	(16)
?	≈5	≈68	A, r	13.6X	(25)

Table Contd.

Table 22.1 Contd.

Sockeye salmon, *O. nerka nerka* (a, m)	≈40	≈90	A, p	2.25X	(8)
(a, m)	54±22.5	≈110	A, p	2X	(7)
(a, f)	136±13.8	≈140	A, p	–	(7)
Coho salmon, *O. kisutch* (j)	≈78	≈140	C, p	1.8X	(4)
(a, f)	141.42.7	227.5±61.5	C, s	1.6X	(24)
Chinook salmon, *O. tshawytscha* (j)	≈20	≈100	A, p	5X	(23)
Bull trout, *Salvelinus confluentus* (j)	8.1±1.2	90±11	A, m	11.1X	(2)
Brook trout, *S. fontinalis* (j)	4.0±0.6	85±11	A, m	21.3X	(2)
Lake trout, *S. namaycush* (j)	2.8±0.4	129±11	A, m	46X	(2)
Arctic charr, *S. alpinus* (j)	≈8	≈40	A, p	5X	(15)
Yellow perch, *Perca flavescens* (j)	8.1±1.2	90±11	A, m	11.1X	(2)
Walleye, *Stizostedion vitreum* (j)	11±4.4	229±16	A, m	20.8X	(2)
Common carp, *Cyrpinus carpio* (j)	7.4±2.9	79±14	A, m	10.7X	(2)
(j)	≈35	≈260	A, p	7.4X	(22b)
Chub, *Leuciscus cephalus* (a)	≈100	1408±154	A, p	14X	(20)
(a)	≈100	810±181	C, p	8.1X	(20)
Walleye Pollock,*Theragra chalcogramma* (j)	144±196	868±197	A, p	6X	(13)
Sablefish, *Anoplopoma fimbria* (j)	7.0±7.4	13.3±22	A, p	–	(13)
(j)	8.4±9.2	169.8±36.1	C, p	20.1X	(13)
Coral reef labrid, *Hemigymnus melapterus* (?)	3	40	A, p	13.3X	(6)
Sea raven, *Hemitripterus americanus* (?)	≈14	≈81	A, m, r	5.8X	(26)
Cod, *Gadus morhua* (a)	≈3	≈27	C, s	9X	(10)

Roach, *Rutilus rutilus*	(2 yr; 5°C)	8.1	400	A, n	49.4X	(19)
	(2 yr; 5°C)	8.1	140	C, p	17.3X	(19)
	(2 yr; 16°C)	1.4	700	A, n	500X	(19)
	(2 yr; 16°C)	1.4	600	C, p	429X	(19)

[1]ND = cortisol not detectable; NA = data not available; ≈ = values extrapolated from figures or averaged from groups

[2]j = juvenile, a = adult, m = male, f = female; ? = stage/sex not stated

[3]A = acute stressor; C = chronic stressor for at least 24 h; m = air exposure; n = capture (netting, hooking); o = oxygen depletion; p = capture and confinement; q = ammonia; r = exercise; s = chasing

[4](1) Avella et al. (1991), (2) Barton (2002), (3) Belanger et al (2001), (4) Davis and Schreck (1997), (5) Fevolden et al. (1993), (6) Grutter and Pankhurst (2000), (7) Kubokawa et al. (1999), (8) Kubokawa et al. (2001), (9) Melotti et al. (1992), (10) Morgan et al. (1999), (11) Norris, D.O. and J. Joss, unpublished observations, (12) Norris et al. (1999), (13) Olla et al. (1997), (14) Orlando et al. (2002), (15) Øverli et al. (1999), (16) Pagnotta et al. (1994), (17) Pankhurst and Dedual (1994), (18) Pottinger and Carrick (2000), (19) Pottinger et al. (1999), (20) Pottinger et al (2000), (21) Ruane et al. (1999), (22a) Ruane et al. (2001), (22b) Sadler et al. (2000), (23) Sharpe et al. (1998), (24) Stratholt et al. (1997), (25) Thomas et al. (1999), (26) Vijayan and Moon (1994).

fish briefly, confining a fish even without handling it, holding fish in high densities or reducing water levels to increase density, or simply feeding the fish (Pickering and Pottinger, 1983; Wedemeyer et al., 1990; Davis et al., 2001).

Prolonged or chronic stress in fishes is typically characterized by loss of body weight, loss of reproductive capacity, and elevated levels of corticosteroids, as reported in other vertebrates (Toates, 1995). Blood clotting time is shortened in rainbow trout by acute stress and the effect is proportional to the severity of the stressor (Ruis and Bayne, 1997). Confinement of rainbow and brown trout (Ruane et al., 1999) or acute injection of cortisol in carp, *Cyprinus carpio* (Wojtaszek et al., 2002) increases hematocrit. Furthermore, chronic elevation of plasma cortisol following implantation of pellets into the body cavity of kokanee salmon (*Oncorhynchus nerka kennerlyi*) increased hematocrit and accelerated blood clotting (Hobbs and Norris, unpublished observations). Under stress, fishes also may show altered behavior such as those involved in food procurement, predator avoidance, courtship, and habitat selection (Olla et al. 1995; Pankhurst and van der Kraak, 1997; Schreck et al., 1997). These altered behaviors may contribute to body weight loss and impaired reproduction.

Measurement of a single parameter related to the HPA axis is insufficient to establish that an animal is chronically stressed. For example, different genetic strains of the same species may exhibit different resting levels of cortisol due to the effects at any level in the HPA axis (Pottinger and Pickering, 1997; Pottinger and Carrick, 1999; Tanck et al., 2001, 2002; Fevolden et al., 2002). Also, two fish populations may have similar plasma cortisol levels, yet one of these population may be experiencing chronic stress. For example, a population of brown trout exposed to heavy metals exhibited identical resting plasma cortisol levels to an upstream population that was not chronically exposed to metals. However, the downstream population exhibited hyperactivity with respect to the number of immunoreactive hypothalamic CRH neurons, the plasma level of ACTH, and the amount of interrenal tissue present when compared to the upstream population not exposed to metals (Norris et al., 1997, 1999). Furthermore, the downstream population could not sustain a normal hormonal response to a chronic stressor (Norris et al., 1999). Is the downstream population stressed with respect to the upstream population? Probably, but plasma cortisol levels alone indicate otherwise.

Approaches to assess chronic stress should examine several parameters of the HPA axis and/or the ability of the animals to mount and sustain a typical stress response following a challenge such as acute confinement (see Wedemeyer et al., 1990; Avella et al., 1991; Norris, 2000).

EFFECTS OF ENDOCRINE-DISRUPTING CHEMICALS ON THE HPA AXIS OF FISHES

Functioning of the fish HPA axis can be altered by anthropogenic chemicals that find their way into aquatic habitats (for reviews, see Colborn et al., 1996; Kendall et al., 1998; Guillette and Crain, 2000). These chemicals have been termed endocrine-disrupting chemicals (EDCs) or contaminants (EDCs) and either mimic natural hormones or block the action of natural hormones. Mimicry can occur by increasing receptor synthesis, binding to and activating the natural hormone's receptor, activating or substituting for an intracellular event that normally occurs after the receptor is occupied by its natural ligand, etc. Other EDCs may decrease the rate of metabolism of a natural regulator, block receptor synthesis or compete for a receptor but not activate it, etc. Many compounds known to be toxic at higher concentrations (e.g., dioxins, polychlorinated biphenyls or PCBs, pesticides, pharmaceuticals, etc.) have been found to exhibit endocrine-disrupting actions at very low doses previously considered 'safe' with respect to overt toxicity as measured by carcinogenic potential or lethality. Nevertheless, these compounds at very low concentrations can affect development, sexual differentiation, and other aspects of physiology and their related behaviors. EDCs are especially troublesome because, at least theoretically, there is no 'threshold' dose since any amount can add to or detract from the action of a natural regulator. Furthermore, they often exhibit u-shaped or j-shaped dose-response relationships with low and higher doses being more active than intermediate doses (Calabrese and Baldwin, 1999). Most scientific attention to date has been on EDCs that disrupt the hypothalamus-pituitary-gonad (HPG) axis, but EDCs also have been found that affect thyroid function (HPT axis) and the HPA axis as well.

Metabolites of the pesticide DDT, such as o,p'-dichlorodiphenyldichloroethane (o,p'-DDD) as well as o,p-DDD and o',p-DDD have been studied in adult fishes with respect to their possible actions on the HPA axis. o,p-DDD reduced circulating cortisol by decreasing the responsiveness of adrenocortical cells to ACTH in a cichlid

fish, *Sarotherodon aureus* (Ilan and Zaron, 1980, 1983). The metabolite o,p'-DDD also depressed cortisol and liver glycogen levels in rainbow trout up to 14 days post-injection (Benguira et al., 2002), whereas o',p-DDD suppressed plasma glucose levels but had no effect on post-stress levels of cortisol or ACTH in Arctic charr (*Salvelinus alpinus*) 30 days after injection (Jørgensen et al., 2001). Another organochlorine pesticide, endosulfan, also reduced ACTH-responsiveness of adrenocortical tissue of rainbow trout *in vitro* (Leblond et al., 2001; Dorval et al., 2003). PCBs also suppress cortisol responses to stress in Arctic charr (Jørgensen et al., 2002) and to ACTH treatment in *O. mossambicus* (Quabius et al., 1997). The response to stress was also impaired in a natural population of yellow perch (*Perca flavescens*) chronically exposed to a mixture of PCBs and metal ions (Hontela et al., 1995).

Adrenocortical tissue from brown trout chronically exposed in rivers to nearly lethal levels of cadmium, as part of a mixture of metals from mining operations, exhibit reduced sensitivity to ACTH compared to adrenocortical cells isolated from fish in a reference population (Norris et al., 1999). These trout showed adrenocortical hyperplasia and evidence of hyperstimulation (nuclear enlargement), increased numbers of CRH-immunoreactive neurons in the hypothalamus (Norris et al., 1997), and blunted responses to a severe stressor (Norris et al., 1999). Cadmium exposure may alter important metabolic enzymes in the liver as well (Norris et al., 2000). Adrenocortical cells from yellow perch (Brodeur et al., 1997) exposed to metals and PCBs in nature were also less responsive to ACTH administered *in vitro*. Similarly, acute *in vitro* exposure to cadmium, mercury, zinc or o,p'-DDD impaired the responsiveness of rainbow trout adrenocortical cells to ACTH (LeBlond and Hontela, 1999).

Adrenocortical cells of chinook salmon (Servizi et al., 1993) or yellow perch (Hontela et al., 1997) showed nuclear enlargement following exposure to bleached kraft mill effluent (BKME). However, other studies of fishes exposed to BKME have not shown consistent effects on adrenocortical function (see Hontela, 1997).

From this brief account, it is evident that a number of different EDCs may alter functioning of the HPA axis in fishes. These effects may be manifest in iono-osmotic or metabolic imbalance, by inappropriate responses to natural or anthropogenic stressors, as impaired disease resistance (see below), or by alterations of major life history events

including development, reproduction, and senescence (see below). Researchers must be aware of potential impacts of these EDCs, especially when studying natural populations. Because of the widespread occurrence of EDCs in aquatic environments (see Kendall et al., 1998; Guillette and Crain, 2000), the realization that no population is free of potential influences of EDCs must be recognized in future experimental designs.

CORTISOL AND IMMUNITY

The immune system and the HPA axis are known to influence each other in mammals. Interleukins from immune cells stimulate HPA activity and cortisol inhibits immune cell functions by inhibiting interleukin secretion and thereby increases sensitivity to infectious agents (see Balm, 1997; Norris, 1997). The high incidence of disease in spawning salmon corresponds to elevated cortisol (Robertson and Wexler, 1960, 1962; Donaldson, 1981) although the cause-effect has not been established experimentally. Similarly, the immune response system of fishes apparently can be inhibited by hyperactivity of the HPA axis induced by physical stressors such as handling and crowding (Mazur and Iwama, 1993a,b) and by infectious agents (Maule et al., 1989). Acute mortalities following exposure to a severe stressor are usually caused by ionic losses, whereas later deaths have been attributed to disease conditions resulting from reduced immunocompetence (see discussion by McDonald and Milligan, 1997). Numerous specific pollutants, including heavy metals (e.g., O'Neill, 1981; Dick and Dixon, 1985; Zellikoff et al., 1995; Dethloff et al., 1999) and BKME (Couillard and Hodson, 1996), and general chemically polluted environments (Weeks et al., 1986; Macchi et al., 1992; Blazer et al., 1994; Rice et al., 1996; see also review by Dunier and Siwicki, 1993) are reported to impair at least one aspect of immune function. Although some studies have found no effect or even stimulatory effects on certain immune parameters (e.g., Thuvander, 1989; Dunier and Siwicki, 1993), the general pattern observed is that of depression of immune competency by activation of the HPA axis regardless of the nature of the stressor and whether the stressor is applied in an acute or chronic manner. Different genetic traits of a species studied in different laboratories and under differing holding conditions may account for some of these observed differences. For example, wild rainbow trout exhibit greater responses to confinement stress and electroshock than do hatchery reared rainbow trout (Woodward and Strange, 1987). Also, the activity of the HPA axis

can be affected by genetic makeup (Pottinger and Pickering, 1997; Pottinger and Carrick, 1999; Tanck et al., 2001, 2002; Fevolden et al., 2002) and by behavioral relationships (e.g., Øverli et al., 1999; Elofsson et al., 2000). Importantly, a change in one immune parameter may not be indicative of the overall effect on immune competency, and multiple markers should always be employed when assessing immune activity. For example, a decrease of leukocytes in blood could be interpreted as a consequence of decreased production (i.e., immune suppression) or could be due to active migration of leukocytes into the tissues related to an active immune response.

CORTISOL AND LIFE HISTORY EVENTS

Cortisol and Development

Although the effects of cortisol on early development have been studied in other vertebrate groups, there are few data available on non-salmonids. There is evidence of maternal transfer of cortisol to the eggs of teleosts, and this transfer is increased from stressed females with elevated plasma cortisol (see Stratholt et al., 1997). Artificially increasing egg cortisol levels by immersion of fertilized eggs in a cortisol solution had no effect on early development or mortality, and maternal or added cortisol was rapidly cleared from the eggs (Stratholt et al., 1997). It is not certain whether or not maternal cortisol is important in the early stages of development, but excessive amounts in the egg do not appear to be detrimental. Studies of reduced cortisol levels in eggs or the presence of GR blockers during early development may provide some insight into whether egg cortisol is simply an insignificant consequence of maternal plasma cortisol levels or whether it plays an important role in development.

Full responsiveness of the HPA axis in rainbow trout does not occur until after hatching: although ACTH stimulates cortisol production from interrenal tissue of unhatched embryos *in vitro*, a cortisol response to a stressor cannot be elicited until two weeks after hatching (Barry et al., 1995). Corticosteroids may influence metamorphosis in fishes. In lamprey, an increase in the activity level of an important steroidogenic enzyme (Δ^5-3β-hydroxysteroid dehydrogenase) in the presumptive adrenocortical tissue occurs prior to metamorphosis (Seiler et al., 1981). In Japanese flounder, *Paralichthys olivaceus*, cortisol levels rise from pre-metamorphosis to the climax, then decline (de Jesus et al., 1991), and cortisol enhances

the effects of thyroid hormones on morphological changes associated with metamorphosis (de Jesus et al., 1990). The possible role of corticosteroids in early development needs to be examined more broadly.

A significant event in the development of salmonid fishes is the process of smoltification, whereby the young fish changes from a cryptically marked, solitary and territorial FW parr to a silvery, gregarious smolt that tolerates SW (see Hoar, 1976, 1988). Smoltification has also been called a second metamorphosis (Youson, 1988) with many hormonal changes and comprehensive biochemical, physiological, morphological, and behavioral changes, as those observed in amphibian metamorphosis (Dickhoff et al., 1990). Secretion of a number of hormones increases during smoltification, including the thyroid hormones, cortisol, prolactin, insulin, and GH (see Dickhoff et al., 1990). Numerous studies implicate thyroid hormones as being responsible for the development of SW tolerance in smolting salmonids (e.g., Nagahama et al., 1982), as well as some morphological changes such as the deposition of guanine in the scales that is responsible for the silvery appearance of the smolts (see Hoar, 1976, 1988). Increased cortisol and probably GH secretion (Specker, 1982; Young et al., 1989) as well as decreased prolactin secretion are correlated with smoltification and adaptation to SW. Migrating chinook salmon exhibited higher cortisol levels than nonmigrants held in still waters (Mazur and Iwama, 1993b; Congelton et al., 2000). Increased ability to osmoregulate in SW is correlated with increases in gill Na^+/K^+-ATPase and chloride cell number (Richman et al., 1987a) and these changes can be induced in hypophysectomized coho salmon by cortisol and GH (Björnsson et al., 1987; Richman et al., 1987b).

Cortisol and Reproduction

Cortisol is usually connected with reproduction through the inhibitory actions of stress on circulating gonadotropins and gonadal steroid hormones (see Pankhurst and van der Kraak, 1997), just as it is in other vertebrates (see Norris, 1997). Stressors may produce differing responses at different stages of maturation or in different species, can affect gamete quality, and can influence later reproduction by their progeny (see Schreck et al., 2001). Stress lowers circulating levels of testosterone and 11-ketotestosterone in male brown trout (Pickering et al., 1987) and estradiol in red gurnard, *Chelidonichthys kumu* (Clearwater and Pankhurst, 1997), and roach, *Rutilus rutilus* (Pottinger et al., 1999). Exogenous

cortisol reduces estradiol levels in female rainbow trout (Pankhurst and van der Kraak, 2000), and suppresses plasma testosterone and estradiol (E_2) levels as well as ovarian growth in tilapia (Foo and Lam, 1993). Although chronic stress altered the timing of reproduction in female rainbow trout and had some effect on certain reproductive parameters, the progeny of the stressed females performed as well as controls with regard to growth and disease resistance (Contreras-Sanchez et al., 1998). In contrast, Morgan et al. (1999) report a higher frequency of abnormal larvae produced by Atlantic cod, *Gadus morhua*, subjected to chronic stress. We might expect widespread species differences in this regard, and additional studies, especially on non-salmonid fishes, are necessary.

Although the effects of stress can be clearly negative on reproduction, corticosteroids may play a positive role in reproductive maturation. Treatment of juvenile female European eels, *Anguilla anguilla*, with cortisol increased production of pituitary gonadotropin (Huang et al., 1999). Furthermore, there is a steady increase in plasma cortisol levels associated with sexual maturation and spawning in salmonids, and these levels often exceed those seen in stressed fish (Pickering and Christie, 1981; Bry, 1985; Kubokawa et al., 1999; Carruth et al., 2000b; Onuma et al., 2003). Although adrenocortical cells from mature female chinook and rainbow trout are more responsive than those of males to ACTH administered *in vitro*, E_2 and 11-ketotestosterone do not seem to have much effect on cortisol synthesis by juvenile or mature fish interrenals *in vitro* (McQuillan et al., 2003). Despite the correlation of increased activity of the HPA axis associated with sexual maturation and spawning, a definite role for cortisol in salmon reproduction has yet to be demonstrated, and this area demands additional examination. The potential actions of EDCs on the HPA axis and possible interactions with reproductive success should be an issue of central focus in future research.

The HPA Axis and Aging

Fishes are as variable in life span as they are in all other aspects of their lives; some live for many years, whereas others complete their entire life cycle in less than a year. Many species show indeterminate growth and continue to grow as long as they live. Senescence is typically not a prominent event observed in the lives of fishes, because sick or weakened fish probably are eliminated by predators. However, with the increasing emphasis on fish aquaculture to meet the food demands of humans,

premature senescence under crowded culture conditions may become an important issue for fish biologists.

A possible connection between the HPA axis and aging has been described for Pacific salmon (several species of the genus *Oncorhynchus*) that exhibit high cortisol levels prior to spawning and death (Phillips et al., 1959; Dickhoff, 1989; Kubokawa et al., 1999, 2001; Carruth et al., 2000b; Onuma et al., 2003). Prior to spawning, there is a marked reduction in circulating levels of androgens and estrogens in the salmon, which is followed by marked tissue degeneration, increased disease, and a heightened susceptibility to infection associated with the continued hypercorticoidism (Robertson and Wexler, 1960, 1962; Donaldson, 1981). Ovulation and spermiation are brought about by a short period of 17,20-dihydroxyprogesterone (DHP) secretion, and DHP levels decline markedly after spawning, while cortisol levels decrease somewhat but remain elevated with respect to immature fish (see Connaughton and Aida, 1999; Carruth et al., 2000b). Some studies indicate higher cortisol levels in spawning female sockeye and chum salmon as compared to males (see Phillips et al., 1959; Kubokawa et al., 2001; Onuma et al., 2003), whereas no sex difference was found in spawning kokanee salmon by Carruth et al. (2000b). Some investigators have suggested the elevation of cortisol and its maintenance at high levels until spawning are associated with energy demands of the stressful migration to the ancestral spawning site (see Dickhoff, 1989). However, landlocked kokanee salmon also exhibit elevated cortisol followed by senescence and death, even though they may migrate only a few miles to their spawning site (Carruth et al., 2000b; Maldonado et al., 2000). Carruth et al. (2002) have proposed that the high cortisol may play a role in recalling imprinted olfactory memories necessary for guiding fish to their spawning sites. In addition, we suggest that chronic, excessive cortisol secretion may be a mechanism to ensure death of the spawned salmon so that decomposition of their bodies will provide the necessary nutrients to the ecosystem necessary for rearing their offspring (see also Gende et al., 2002). Chronic administration of cortisol to young rainbow trout (Robertson et al., 1963) or kokanee salmon (Hobbs and Norris, unpubl. data) causes degenerative changes and can induce death.

In the brain of both kokanee salmon and a cichlid fish, *H. burtoni*, CR are localized in many discrete areas including sensory regions (e.g., the internal layer of the olfactory bulb, the putative hippocampus,

glomerulosus complex of the thalamus), in the Purkinje cells of the cerebellum, and in the NLT of the hypothalamus (Teitsma et al., 1997, 1999; Carruth et al., 2000a, 2002). Studies using *in situ* hybridization (Teitsma et al., 1997) and immunocytochemistry (Teitsma et al., 1999) show that the highest density of GR receptors occurs in two areas of the diencephalon of *H. burtoni* that control the HPA axis: magnocellular preoptic region and the mediobasal hypothalamus.

With sexual maturation and spawning, there is a translocation of CR from cytoplasm to nucleus in some of these regions (Carruth et al., 2000a), and this translocation can be induced in immature kokanee by the injection of cortisol (Carruth et al., 2002). Neurodegeneration is accelerated in most of these CR-positive areas just prior to spawning and this is accompanied or followed by the deposition of beta-amyloid (Aβ) protein in neurons and in extracellular plaques similar to those observed in mammals (Maldonado et al., 2000, 2002a,b; see Fig. 22.3). Although mammalian studies have verified that high cortisol can cause neurodegeneration (see Sapolsky et al., 1985, 1986; Jacobson and Sapolsky, 1991; McEwen et al., 1999; Sapolsky, 1999), more studies are needed to ascertain whether cortisol is responsible for the accelerated neurodegeneration and Aβ deposition preceding the death of Pacific salmon.

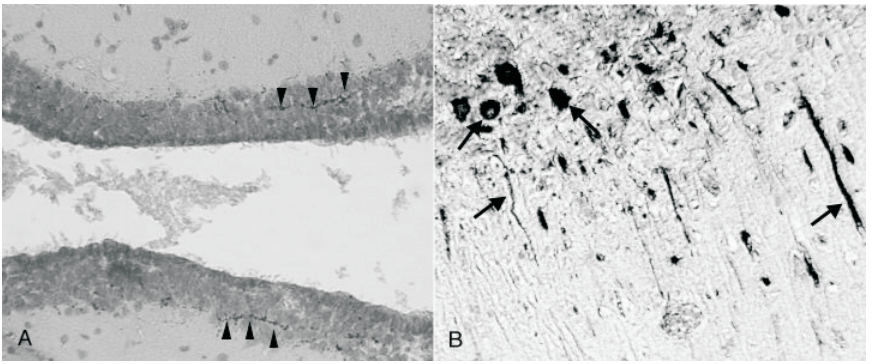

Fig. 22.3 Aβ deposits in the periventricular zone of the ventral hypothalamus (A), and intracellular Aβ in the cerebellum (B) of a sexually immature cortisol-treated kokanee salmon. The Aβ deposits appear distributed along the length of an axon or dendrite (arrowheads). The absence of clear extracellular deposits and the appearance of cytoplasmic, and dendritic Aβ in the cerebellum (arrows), suggests that intracellular Aβ aggregation may seed extracellular plaque formation following cell lysis. The distribution of Aβ parallels cortisol-responsive brain regions in spawning salmon. Photograph by Steven Hobbs, 2003.

The appearance of protein aggregates late in life and, following chronic cortisol elevation, also suggests a possible connection with hsps. As was previously discussed, cortisol signaling through CRs downregulates some hsps, and hsps are critically important for preventing protein aggregation. Aβ is also known to interact intracellularly with hsps (Fonte et al., 2002). Taken together, the evidence suggests that intracellular Aβ aggregation may result from cortisol mediated attenuation of hsps. When the Aβ-laden neurons degenerate, possibly due to cortisol and/or Aβ toxicity, or for other reasons entirely, the release of Aβ into the extracellular space may seed plaque formation.

CONCLUSION

The HPA axis, involving the hypothalamus, the pituitary, and the adrenocortical tissue, is critical to the normal functioning of fishes. Cortisol, the principal corticosteroid secreted in fishes, interacts with a variety of specific receptor types (both MR and GR types as well as splice variants) to produce its characteristic effects in target tissues. Occupied cytosolic corticosteroid receptors complexed with certain heat shock proteins regulate genomic expression in target cells. Cortisol plays essential roles in development, stress responses, metabolism, osmoregulation, reproduction, and immunity. Furthermore, excessive levels of cortisol may be responsible for neurodegeneration in aging fishes. In addition, the health of the HPA axis in fishes may be especially sensitive to a variety of endocrine-disrupting chemicals of anthropogenic origin.

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CHAPTER

23

Modes of Action and Physiological Effects of Thyroid Hormones in Fish

J. Geoffrey Eales

ABSTRACT

Experimental approaches for increasing or decreasing thyroidal status to determine the roles of thyroid hormones in fish have been examined. Despite some problems in design or interpretation, experiments in the last half century show that thyroidal status influences many processes, including growth, development, parr-smolt transformation and reproduction. In most instances, thyroid hormones exert a permissive role. Thyroid status also depends on the anabolic or catabolic state and a threshold thyroidal status may play a limiting role in allowing other hormones to exert their directive effects under metabolically-optimal conditions. Despite considerable recent interest in effects of Environmental contaminants on the fish thyroid, the extent of their impact on thyroidal status and its physiologically-dependent end-points is unclear.

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INTRODUCTION

Although many effects have been attributed to thyroid hormones (TH = thyroxine (T4) and 3,5,3'-triiodothyronine (T3)) in fish, the modes of TH action are still not fully understood. Three traditional approaches have been used in the numerous studies on this topic: (i) correlations between physiological or environmental changes and thyroid end-points, (ii) experiments altering environmental or physiological states followed by observations of changes in thyroid end-points, and (iii) experiments altering thyroid status (availability of TH to target tissues) followed by observations of physiological end-points. All three approaches have been helpful but the last approach is the only one to provide definitive answers on TH action and is the one emphasized in this review. However, even hypothesis-based experiments may be difficult to interpret due to their design. Ambiguities arise either due to the methods for manipulating thyroid status or to the complex adjustments these manipulations induce in the thyroid system itself. This chapter is, therefore, prefaced with consideration of these two aspects. The TH effects proposed for fish are too diverse for comprehensive treatment and so this coverage is restricted to TH roles in somatic growth, early development and metamorphosis, parr-smolt transformation and reproduction. Finally considered are possible impacts of environmental contaminants.

METHODS FOR EXPERIMENTALLY MANIPULATING THYROIDAL STATUS IN FISH

TH injection is widely used. It delivers precise amounts of TH into the fish but creates spikes in plasma TH levels that then decline until the next injection. Kinetic analysis of TH in trout at 12°C showed a mean system residence time of 13 - 22 h for T4 and 35 - 52 h for T3 (Sefkow et al., 1996). The relatively short T4 residence is consistent with observations of high initial post-injection plasma T4 levels and then rapid clearance. For example, intraperitoneal injection of 18ng/g initially raised trout plasma T4 27-fold but by 20 h control T4 levels were reestablished (Eales, 1974). An unstudied issue is the effect of injection site on subsequent systemic TH availability. For example, the commonly-used intra-peritoneal

injection may cause high TH levels in certain visceral tissues and promote local metabolism of TH prior to their systemic access.

Food represents a non-invasive vehicle for administering T3. A single meal of 12 mgT3/kg (ppm) raised coho plasma T3 levels 3-fold by 12 h but by 23 h control levels were reinstated (Sullivan et al., 1987a). However, chronic feeding at 5-15 ppm T3 in food usually achieves predictable and relatively consistent physiologic 2–3-fold elevations in plasma T3 with exact levels depending on species, food ration and meal frequency (e.g., Higgs et al., 1982; Eales et al., 1990; Mackenzie et al., 1993). Surprisingly, this method is far less effective with T4. Indeed, T4 feeding by an identical protocol did not alter plasma T4 levels in red drum (Mackenzie, 1993) and the massive dose of 1000 ppm fed to several salmonids for 3 days only raised the plasma T4 to ~30 ng/ml (Hutchinson and Iwata, 1998). The generally weak response of plasma T4 to dietary T4 may be due to a combination of T4 negative feedback on the brain-pituitary-thyroid (BPT) axis to reduce endogenous T4 secretion, relatively rapid plasma T4 clearance (Sefkow et al., 1996), rapid T4 degradation (Eales and Brown, 1993) and poor T4 absorption from the intestinal lumen (Whitaker and Eales, 1993). However, Kohel (2004) found comparable T4 and T3 absorption from the trout intestine but a greater degradation of T4 by the intestinal tissues themselves. This may protect systemic blood from T4 surges due to natural ingestion of T4 (Eales, 1997).

TH are absorbed across the gills from the ambient water providing a non-invasive delivery. A nominal ambient level of 5-10 μg T4/100 ml sustained physiologic elevations in plasma T4 in several salmonid and catfish species (Eales, 1974). A steady-state between water and plasma T4 was reached by 4h, indicating rapid adjustment by the BPT axis through negative feedback to maintain a new artificial steady state. However, the plasma responses to ambient T3 are somewhat different. In parallel experiments with ambient T3, plasma T3 levels required up to 24 h to attain steady-state levels at about 10% of ambient concentration (Eales and Collicut, 1974; Omeljaniuk and Eales, 1985). Interestingly, at low T3 concentrations, plasma T3 was greatest before the steady state was attained, suggesting a lag in the induction of peripheral T3 autoregulation. Below 0.125 μg T3/100 ml there was no saturation of trout hepatic nuclear T3-binding sites (putative receptors) but at 0.25 μg T3/100 ml there was partial saturation and at 1.25-3.8 μg T3/100 ml complete saturation (Omeljaniuk and Eales, 1985).Thus for this system a physiologic working range encompassed ambient levels of ~ 0.1-5. 0 μg T3/100ml.

Injecting thyroid stimulating hormone (TSH) also enhances thyroid status. Mammalian or fish TSH forms raise teleost plasma T4 levels within 1h and usually without altering plasma T3 levels, indicating T4 as the primary secreted TH (Eales and Brown, 1993). In the parrotfish *in vitro* thyroid model, T4 was secreted within 20-30min of TSH addition to the perfusate (Grau et al., 1986). Pharmacologic injected TSH doses elevated trout plasma T4 for 48h but at lower doses the response was transient (Chan and Eales, 1976). This is consistent with rapid TSH and T4 plasma clearances and with the observed highly transitory natural surges in plasma T4. However, high TSH doses can cause pathologic cell swelling and follicle rupture (Smith and Grau, 1986) and may explain some of the bi-phasic response to TSH by the fish thyroid (Chan and Eales, 1976; Nishioka et al., 1987). Teleost responses to TSH may depend on stage of development, season, photoperiod, temperature and salinity (Eales and Brown, 1993). Chronic fasting blunted the response to TSH in trout (Leatherland and Farbridge, 1992). However, under appropriate conditions TSH can be used to create precisely-timed acute physiologic elevations in plasma T4 without appreciable change in plasma T3.

Reduction in thyroid status by surgical or radiochemical thyroidectomy is generally impractical in fish whose thyroid function is more effectively blocked by chemicals that competitively or non-competitively create lesions within the thyroid hierarchy and mainly on thyroid tissue itself. Therefore many of these inhibitors may only be effective after a fully-formed thyroid has developed. They include thiourea (TU), perchlorate, thiouracil and propylthiouracil (PTU). Methimazole was also shown recently to be effective (Alexander et al., 1998). In mammals, PTU also inhibits certain TH deiodination pathways. This effect appears weak in fish (Shields and Eales, 1986; Sanders et al., 1997), although Ebbesson et al. (1998) described possible PTU inhibition of deiodination in coho smolts. Ipodate and iopanoic acid are probably more effective inhibitors of extra-thyroidal deiodination (Spieler and Noeske-Hallin, 1985; Cyr and Eales, 1986; Leloup and Lebel, 1993). The above inhibitors have been injected in solution or suspension, implanted in silastic tubing or added to the ambient water in either open or closed systems. A major concern is inhibitor specificity and hence the potential for side effects. Apparent extra-thyroidal effects of low TU doses to increase hematocrit and to shift water and electrolyte balance occurred in

trout (Eales, 1981). Phenylthiourea inhibited cichlid melanogenesis and tyrosinase activity without linkage to thyroidal changes (Pietzsch-Rohrschneider, 1977; Lanzing, 1984). PTU suppressed growth and altered several metabolic parameters in coho salmon without depressing the plasma TH levels (Sullivan et al., 1987a). To allow for confounding extra-thyroidal effects, inhibitor-treated fish should be compared with inhibitor-treated fish also treated with TH in order to reinstate TH availability to target tissues, but relatively few workers have used this additional control. Furthermore, thyroid status should be checked after inhibitor treatment as not all potential inhibitors are as effective in fish as anticipated (Milne and Leatherland, 1978; Eales, 1981; Eales and Omeljaniuk, 1981; Eales and Shostak, 1983; Sullivan et al., 1987a). Indeed, KSCN is toxic to trout at concentrations that do not block thyroid function (Eales and Shostak, 1983), likely due to its conversion to cyanide (Heming et al., 1985).

All of the above techniques are applied *in vivo* but several *in vitro* approaches have also been used. These have involved TH treatment of cultured cells (mainly primary cultures) or subcellular organelles followed by measurement of physiologic end-points. The advantage of this approach is that direct effects on a putative target tissue can be tested under precisely maintained conditions. Such studies have shown TH effects on insulin-like growth factor (IGF) synthesis by tilapia hepatocytes (Schmid et al., 2003), growth hormone (GH) secretion by eel pituicytes (Luo and McKeown, 1991; Melamed et al., 1995; Rousseau et al., 2002), deiodination by trout hepatocytes (Sweeting and Eales, 1992), lipid metabolism in eel hepatocytes (Ndiaye and Hayashi, 1997), and 17-β-estradiol (E2) secretion by trout oocytes (Cyr and Eales, 1988). Rapid TH effects on isolated mitochondria have also been reported (Arsan et al., 1987; Peter and Oommen, 1988; Leary et al., 1996). Physiologic doses of TH are difficult to determine for *in vitro* studies, particularly where the medium lacks TH-binding proteins to buffer the free T3 level. Reported effective nominal T3 concentrations usually range from 10 to 30 nM, corresponding to physiologic total plasma TH levels but probably exceeding *in vivo* free TH levels.

In summary, there are numerous *in vivo* and *in vitro* methods available for acutely or chronically manipulating thyroid status of fish. Regardless of the method, an obvious objective is to create physiologically meaningful changes in thyroid status with the minimum possibility of untoward side effects. These ideals can be challenging to attain. Physiologic TH doses

can be particularly difficult to prescribe and may be species- and stage-dependent. For example, in salmonids for most of their life a doubling or tripling of plasma T4 would be physiologic but at parr-smolt transformation much higher natural transitory surges in plasma T4 occur.

THYROID RESPONSES TO MANIPULATION OF TH AVAILABILITY

During its long evolution, the thyroid system has acquired homeostatic mechanisms to offset disruptive conditions, such as iodine deficiency or a dietary TH excess, that might otherwise compromise its normal function. These same mechanisms will be brought into play when the thyroid status is experimentally manipulated and it is crucial to consider their impact on the thyroid system, particularly following TH supplementation. TH are typically administered either as T4 or T3 and they are considered separately below since, to a large degree, they are thought to be independently regulated in fish (Eales and Brown, 1993).

Effects of Exogenous T4

A long-established effect of an exogenous T4 challenge is to suppress endogenous T4 secretion via negative feedback on the BPT axis. This may be considered of no consequence in experimental design as exogenous T4 is given specifically to raise plasma T4 levels, regardless of the source. However, reduction in endogenous T4 secretion and replacement with excess exogenous T4 will tend to eliminate the pronounced naturally-occurring short-term fluctuations in plasma T4 characteristic of teleosts. Of particular significance will be attenuation of the daily cycle of plasma T4 shown for several species (e.g., Spieler and Noeske, 1981; Cook and Eales, 1987; Boujard and Leatherland, 1992; Pavlidis et al., 1997). This cycle has been shown most convincingly for red drum in which there is a bimodal, free-running and photoperiod-independent cycle with a periodicity uninfluenced by food intake (Leiner et al., 2000; Leiner and MacKenzie, 2001). The robustness of this endogenous cycle implies its physiological significance. Furthermore, plasma T4 levels may be influenced by melatonin (Gupta and Premabati, 2002) and also respond acutely to 'non-cyclic' stressful stimuli and changes in blood glucose levels (Brown et al., 1978; Himick and Eales, 1990). In addition, highly transient natural changes in plasma T4 occur in migrant juvenile salmonids. While the underlying stimuli and mechanisms may vary with circumstance and

species, all the above examples show that the set-point of the teleost BPT axis adapts rapidly to regulate short-term plasma T4 fluctuations, which presumably have physiologic significance. Current methods of T4 treatment will either eliminate or mask these short-term changes in plasma T4 or impose a new cycle based on the frequency of T4 administration. The influence on physiologic end-points is unclear.

Since T4 is the immediate precursor of T3 through T4 outer-ring deiodination activity in peripheral tissues, it would be expected that a T4 challenge would, through added availability of T4 substrate, drive the enyzmatic production of T3 and also elevate plasma T3 levels. However, following injection of T4 or TSH, there can be massive increases in plasma T4 with no change in plasma levels of T3 (Eales and Brown, 1993). Furthermore, 'constancy' in plasma T3 is also evident from the negligible, or greatly dampened, natural fluctuations in plasma T3 accompanying the previously-described more pronounced natural fluctuations in plasma T4. This relative stability in plasma T3 stems, at least in part, from 'immediate' buffering and longer-term regulation.

In contrast to T4, T3 exchanges rapidly with trout erythrocytes which carry a significant proportion of total circulating T3 and, hence, buffer plasma T3 levels (McCleese et al., 1998). However, slowly-exchanging skeletal muscle, sequestering about 80% of T3 in trout, is probably the main buffer (Fok et al., 1990; Sefkow et al., 1996). Regulation mainly involves changes in activities of the deiodinases, removing iodine from either the outer ring (outer-ring deiodination = ORD) or the inner ring (inner-ring deiodination = IRD). Upon T4 or T3 challenges the T4ORD pathway forming endogenous T3 from T4 is suppressed, while the T4IRD pathway degrading T4 to inactive rT3 and the T3IRD pathway degrading T3 to 3,3'-T2 are induced (Eales and Brown, 1993; Morin et al., 1995; Mol et al., 1999). These deiodination adjustments have been most studied in liver where they likely contribute to systemic T3 autoregulation but they also occur in brain, retina, kidney, skin and intestine (Byamungu et al., 1990, 1992; Morin et al., 1995; Fines et al., 1999; Plate et al., 2002; Plohman et al., 2002b; Kohel et al., 2004), where they may regulate T3 levels in individual target tissues. Intestinal deiodination may be especially important in regulating plasma TH levels in the face of natural or experimental dietary TH challenges.

In summary, based on data available to this point and, in contrast to the situation in mammals, increasing amounts of T4 do not drive T3

production and, hence, do not lead to increased availability of T3 to target tissues from the plasma T3 pool. Thus, if T4 administration changes physiologic end-points, it achieves this either by acting as the effective ligand for the thyroid receptors (TRs) or by its local conversion to T3 in target tissues. Based on the extensive autoregulation of T3 by deiodination in many tissues, the latter possibility is unlikely and so, in T4-treated fish, the presumed natural ligand, T3, may play a minor role. Furthermore, this will take place in the absence of the normal diurnal cycle in plasma T4 levels. At present, the consequences of these abnormalities on the interpretation of physiologic end-point effects remain unclear.

Effects of Exogenous T3

The main effect of T3 administration is to raise the plasma and tissue T3 levels at the expense of endogenous T3 whose production decreases due to autoregulation by adjustments in deiodination pathways. A major point of contention is whether the elevated plasma T3 level also exerts a negative feedback action on the BPT axis to depress the plasma T4 level. In many instances, T3 did not change the plasma T4 level (de Luze and Leloup, 1985; Lin et al., 1985; Iwata et al., 1989; Eales et al., 1990) and, in a few instances, raised it (Howerton et al., 1986; Farbridge and Leatherland, 1988). In contrast, T3 negative feedback has been inferred either from depressed plasma T4 levels and histological inactivation of the thyroid (Higgs et al., 1979, 1983; Fagerlund et al., 1980; McBride et al., 1982; Rivas et al., 1982; Sullivan et al., 1987a; Eales et al., 1990; Shelbourn et al., 1992; Larsen et al., 1997; Pradat-Balade et al., 1999; Leiner and MacKenzie, 2003) or from *in vitro* inhibition of TSH b-subunit expression (Pradat-Balade et al., 1997; Schmitz et al., 1998; Sohn et al., 1999). However, there exists a number of alternate explanations.

Firstly, most T3-feedback actions reported for fish have involved high or prolonged T3 administration. Based on the mammalian model, negative feedback by T4 likely involves T4 uptake by thyrotropes that locally convert T4 to T3, which then binds to thyrotrope nuclear TRs. Thus the extracellular signal is T4 but the intracellular signal is T3. Therefore, a sufficiently high or prolonged T3 exposure may be guaranteed to elicit negative feedback action due to thyrotrope uptake of lipophilic T3. At issue is whether T3 at physiologic levels enters feedback cells to the extent that it can exert a short-term action on the BPT axis comparable to that exerted by T4.

Secondly, following T3 administration, there will be a surfeit of T4 due to suppression of the T4ORD pathway (Eales and Brown, 1993). Thus, T3 would be expected to exert a negative feedback on the BPT axis by this indirect route and this could explain the histological changes seen in the thyroid. Finally, T3 also induces the T4IRD pathway degrading T4 to rT3 (Eales and Brown, 1993). This will promote plasma T4 clearance and may also tend to depress plasma T4 and give the impression of T3 negative feedback.

Increased T3 levels might also downregulate TRs as shown by their maximum binding capacity (MBC). Few data are available. In rainbow trout and coho salmon, TR MBC was unaltered by a chronic elevation in plasma T3 (Darling et al., 1982; Sullivan et al., 1987a; Bres et al., 1990). However, following either *in vivo* or *in vitro* treatments with T3, the MBC of perch ovarian nuclei was increased (Maitra and Bhattacharya, 1989).

In summary, the main effect of exogenous T3 is to increase plasma and tissue T3 levels at the expense of endogenously formed T3. Its somewhat controversial effects on plasma T4 are relatively minor and may involve a combination of central and peripheral adjustments. Exogenous T3 does not appear to downregulate T3 receptors and, in most instances seems to be the most reliable means of elevating thyroid status but with some possible blunting of the diurnal T4 cycle (Leiner and Mackenzie, 2003). However, to simulate the surges in thyroidal status at parr-smolt transformation T4, rather than T3, might be the administered TH of choice as there is little evidence of an elevation in plasma T3 levels at this time.

Effects of TH Deprivation

Most thyroid inhibitors are administered to either decrease thyroidal T4 secretion (T4 availability) or extra-thyroidal conversion of T4 to T3 and, ultimately, T3 availability. The compensatory adjustments to inhibitors have received little attention. Many inhibitors act as goitrogens in the sense that they stimulate thyroid tissue function by presumed increased TSH secretion resulting from negative feedback relaxation. To what extent this offsets the effect of the inhibitor will depend on treatment dosage and duration. Short-term treatments may be ineffective either due to the thyroid reserve of T4 or to compensatory autoregulation of peripheral deiodinating systems leading to more effecient use of T4 substrate to maintain T3 levels (Eales and Brown, 1993; Mol et al., 1999).

Ipodate, which depresses plasma T3, lowered the TR MBC but this effect could not be reversed by T3 treatment suggesting that the downregulation by ipodate was probably not due to T3 deprivation (Bres et al., 1990). PTU decreased TR MBC of coho salmon but was not tested along with T3 (Sullivan et al., 1987a). Thiourea or thiouracil depressed the TR MBC of perch ovarian nuclei (Maitra and Bhattacharya, 1989).

In summary, experimental changes in TH availability influence the fish thyroid hierarchy at several levels. Effects range from the rapid negative feedback of T4 (and possibly T3) through the BPT axis to suppress diel T4 cycles to the usually slower induction or repression of specific deiodination pathways that may regulate T3 availability in individual target tissues. These adjustments reflect the underlying homeostasis which allows the naturally-operating thyroid system to autoregulate under conditions that might otherwise compromise its function—e.g., conditions of iodine deficiency or a dietary TH excess. Consequently, experimental changes in TH availability, particularly if chronic and at high levels, may have effects other than those anticipated solely on the basis of routinely-measured plasma T4 and T3 levels. How these adjustments influence interpretation of TH actions remains to be demonstrated but they should not be disregarded and may differ for each experimental protocol.

MODE OF TH ACTION AT THE SUBCELLULAR LEVEL

Genomic Actions

Early studies of fish TRs examined ligand-binding and other properties of nuclei or nuclear extracts from potential target tissues (Eales and Brown, 1993). The putative fish TRs closely resemble their mammalian counterparts. They reversibly bind natural TH ligands (affinity for T3 ($K_a \sim 10^9$ liters. mol^{-1}) >T4>>>>r T3), with certain analogues (TRIPROP> TRIAC > TETRAPROP) binding more avidly than T3. TRs of trout and Arctic charr pituitaries had higher T3 affinities than those of other tissues (Bres and Eales, 1989). In trout, the MBC varied widely between tissues (liver > gill> kidney >brain> erythrocytes >> spleen (Sullivan et al., 1987b; Bres and Eales, 1988; Lebel and Leloup, 1989). Ovarian and testicular (Leydig cell) TRs have been reported in perch (Chakraborti et al., 1986; Maitra and Bhattacharya, 1989; Bandyopadhyay and Bhattacharya, 1994). Kudo et al. (1994) reported TRs in the olfactory system and brain of masu salmon.

There is some evidence for regulation of TR MBC due to physiologic state. Although trout hepatic MBC was unaltered by growth rate, diet or food ration it was inversely correlated with fish size and was depressed by E2 or fasting for 3 or 12d (Bres et al., 1990; Eales et al., 1990). Darling et al. (1982) also found reduced hepatic MBC in fasted coho salmon. Sullivan et al. (1987b) reported a higher MBC in trout pro-erythrocytes than in mature erythrocytes. MBC was highest in perch ovarian nuclei at pre-spawning (Maitra and Bhattacharya, 1989; Bandyopadhyay and Bhattacharya, 1994). Seawater adaptation of brown trout elevated the branchial MBC 3-fold (Leloup and Lebel, 1993). The sea lamprey hepatic MBC was higher in metamorphosing larvae than in young adults or upstream migrating adults (Lintlop and Youson, 1983). However, acclimation over the range 5-19°C had no influence on the hepatic MBC (or K_a) of trout (Eales et al., 1986).

Recent molecular cloning and characterization of TRs in several taxonomically diverse teleosts have shown α and β forms homologous to those of other vertebrates (Yamano et al., 1994; Yamano and Inui, 1995; Essner, 1997; Ro et al., 1998; Yamano and Miwa, 1998; Llewellyn et al., 1999; Liu et al., 2000; Marchand et al., 2001, 2004; Nowell et al., 2001; Power et al., 2001; Jones et al., 2002; Kawakami et al., 2003a, b). In mammals, there is a single TRα and a TRβ1 and a TRβ2 but the two βs are due to RNA splice variation. In contrast, several forms of both α and βTRs exist in teleosts. Furthermore, depending on species, not all the α and β forms are necessarily splice variants but may be produced by two α genes or two β genes. The tissue distribution and changes in abundance of these receptor forms during development has suggested potential target tissues for TH but the biochemical and ligand-binding properties of these various TRs are only beginning to be understood (Power, 2001) and the subcellular events following the presumed TH-TR association remain largely unstudied in fish. However, there is some evidence that, as for the established tetrapod model, the TH-TR subunits bind to short sections of DNA (thyroid response elements) either as homo-dimers with a similar TH-TR subunit or as hetero-dimers with a different ligand-receptor subunit (Power et al., 2001). The entire complex may then act as a transcription factor to stimulate or repress RNA synthesis by a nearby gene and thereby regulate specific protein synthesis. Of particular interest will be the ability of fish TRs to form heterodimers with retinoic acid receptors (Alsop et al., 2004), as retinoic acid also influences fish development.

Non-genomic Actions

These have been proposed for mammals (Davis and Davis, 1996; Hulburt, 2000) but there are few studies in fish. Cyr and Eales (1989) observed a rapid cycloheximide-independent, but cAMP-dependent, T3 enhancement of GTH-induced E2 secretion by trout oocytes that may be explained by a decrease in phosphodiesterase activity. Direct *in vitro* TH stimulation of succinate dehydrogenase activity of fish liver mitochondria has been reported (Arsan et al., 1987; Peter and Oommen, 1989). High TH levels were used but TH did reverse the inhibition found in mitochondria from thiouracil-treated fish and the effect was blocked *in vitro* by inhibitors of protein synthesis, suggesting that TH might directly stimulate mitochondrial protein synthesis (Peter and Oommen, 1989). Shivakumar and Jayaraman (1986) described a direct action of T4 on gill mitochondria. Leary et al. (1996, 1997) reported tissue- and substrate-specific direct effects of T3 and 3,5-T2 on state-3 and state-4 respiration after a 5-min incubation with goldfish mitochondria.

In summary, genomic TRs resembling those of other vertebrates have been established for teleosts. Several α and β forms exist. The subcellular events following TH-TR interaction have been little studied but likely conform to those described for higher vertebrates. There is some evidence for rapid non-genomic TH actions.

TH EFFECTS

Somatic Growth

TH can enhance teleost somatic growth (Higgs et al., 1982). The effect is most compelling in young salmonids (Higgs et al., 1982) but also occurs in some other species (Matty and Lone, 1985; Howerton et al., 1986; Degani and Dosoretz, 1986; Degani and Gallagher, 1986; Woo et al., 1991; Ansal and Kaur, 1998). TH also promote growth-associated processes including appetite, food conversion efficiency and activities of intestinal enzymes (Higgs et al., 1982; Woo et al., 1991), RNA levels, amino acid incorporation into protein and protein levels in tissues such as liver and muscle (Eales, 1979; Bhattacharya et al., 1985; Matty and Lone, 1985; De et al., 1989a), cartilage and bone growth (Takagi et al., 1994), scale growth (Shinobu and Mugiya, 1994) and age-related changes in muscle myosin forms (Martinez et al., 1995). TH are usually given in food and T3 is far more effective than T4. However, skeletal and other abnormalities appear

in salmonids above 10-15ppm T3 (Higgs et al., 1982; Hilton et al., 1987). Abnormalities also arise in non-salmonids in which growth may not be enhanced by TH (Matty and Lone, 1985; Gannam and Lovell, 1991; MacKenzie at al., 1993; Moon et al., 1994). Thus, while for some species (e.g. salmonids) there is scope for low doses of exogenous T3 to promote anabolism, for other species endogenous T3 levels may already be optimal and not enhanced by exogenous T3 which may, instead, be ineffective, catabolic or otherwise detrimental (Matty and Lone, 1985; MacKenzie at al., 1993; Valente et al., 2003).TH actions on fish growth are, therefore, complex and depend on species and various *exogenous* and *endogenous* factors.

Exogenous factors can include temperature, diet, feeding frequency, photoperiod, stressors and age (McBride et al., 1982; Matty and Lone, 1985; Scott-Thomas et al., 1992; Martinez et al., 1995; Ansal and Kaur, 1998). Food quantity and quality may be particularly critical as they affect endogenous thyroid status (Eales, 1988; Riley et al., 1993, 1996; MacKenzie et al., 1998; Nankervis et al., 2000). Thyroid status, as judged by deiodination activity promoting T3 availability, increased with total caloric intake and, at least in piscivorous salmonids, with the protein rather than the lipid or carbohydrate content (Eales and Brown, 1993). Often overlooked is the TH content of the control diet itself (Moon et al., 1994; Eales, 1997).

Endogenous factors include anabolic or catabolic growth-relevant hormones (GH, IGFs, glucocorticoids (cortisol) and sex steroids) with which TH may interact to influence the growth rate. Potential interactions exist at the level of the target tissue themselves but the systemic interactions are better studied. Of particular interest is the interplay between the thyroid and GH/IGF systems (see: Reinecke, this volume). GH injection increases thyroid status in the eel (de Luze and Leloup, 1984) and salmonids (MacLatchy et al., 1992) by increasing plasma T3 levels through increased hepatic T4ORD activity, although in GH-transgenic fish, decreased hepatic T3 degradation was the main mechanism (Eales et al., 2004). In contrast, in tilapia, catfish and carp, GH did not stimulate the thyroid system and somatostatin enhanced renal deiodination of T4 to bioinactive rT3 (Byamungu et al., 1990). Studies on the reciprocal effect of TH on GH function reveal that T3 promoted GH release and/or GH gene transcription in trout (Luo and McKeown, 1991; Moav and McKeown, 1992), while T3 *in vitro* increased GH synthesis by

tilapia pituitaries (Melamed et al., 1995) and PTU lowered plasma GH levels in coho salmon (Ebbesson et al., 1998). However, *in vivo* and *in vitro* administration of T3 inhibited eel GH synthesis and release, indicating a possible negative feedback action (Rousseau et al., 2002). Both *in vitro* and *in vivo* experiments show that T3 increases hepatic IGF-I mRNA expression in tilapia (Schmid et al., 2003).

Cortisol and TH interactions have focused on cortisol effects on the thyroid. A few studies suggest that cortisol has no effect or enhances thyroid status (Leatherland, 1987b; Vijayan et al., 1988) but in general cortisol lowers thyroid status by reducing plasma T4 responses to TSH (Leatherland, 1987b) and circulating TH levels (Redding et al., 1986; Weisbart et al., 1987; Vijayan and Leatherland, 1989). Cortisol or its analogue, RU486 (Vijayan and Leatherland, 1992; Reddy et al., 1995) was reported to depress salmonid hepatic deiodination of T4 to T3 but Vijayan et al. (1988) also reported that cortisol increased hepatic T4 deiodination. These latter reports are inconsistent with that of Brown et al. (1991) who found that cortisol enhanced trout plasma T3 clearance and depressed plasma T3 without changing hepatic T4 to T3 conversion. Since stressors induce the trout hepatic IRD pathways that degrade T3 to T2 (Johnston et al., 1997; Todd and Eales, 2002), the cortisol-induced increase in plasma T3 clearance and fall in plasma T3 most likely reflect greater T3, rather than T4, deiodination and degradation.

Sex steroid effects on the thyroid system are complex and depend greatly on species and stage in the sexual cycle. In summary (Cyr and Eales, 1996), the androgens are frequently anabolic, tending to enhance thyroidal status as shown by increased plasma T3 levels, T4 clearance and hepatic T4ORD activity. In contrast, E2 tends to depress plasma T3 levels and lower thyroidal status by several mechanisms. This may contribute to somatic growth arrest in those species where considerable energy reserves are used for gonadal growth. (See Reproduction)

In summary, TH influence growth but the mechanism is poorly understood. TH act in concert with several other hormones and may exert a mainly permissive role. Negative effects of TH may reflect an endogenous thyroidal status already optimal for growth.

Early Development and Metamorphosis

TH influence development and metamorphosis in many teleosts, although in early development, the absolute need for TH is equivocal (reviews by

Youson, 1988; Brown and Nunez, 1994; Power et al., 2001, and more recent studies by Soffientino and Specker, 2001, 2003; Gavlick et al., 2002; Jones et al., 2002; Kawakami et al., 2003a, b). The BPT axis activity is greatest in flatfish at metamorphic climax (Miwa and Inui, 1987) and in many studies thyroid inhibition impairs aspects of early development or metamorphosis while TH administration enhances or accelerates them, though some studies have given contradictory results (Power et al., 2001). Furthermore, plasma or whole-body TH levels or deiodination activity are correlated with certain developmental phases (Tagawa and Hirano, 1987, 1990; Tanagonan et al., 1989; Tagawa et al., 1990; de Jesus et al., 1991; Yamano et al., 1991; de Jesus and Hirano, 1992; Kimura et al., 1992; Reddy et al., 1992). Finally, molecular cloning techniques have revealed tissue sites of putative TR α and β forms and shown changes in their relative and absolute abundances that can also be correlated with developmental stages (Yamano et al., 1994; Yamano and Inui, 1995; Essner, 1997; Llewellyn et al., 1998, 1999; Yamano and Miwa, 1998; Liu et al., 2000; Marchand et al., 2001, 2004; Nowell et al., 2001; Jones et al., 2002; Kawakami et al., 2003a, b). These correlations imply changes in thyroid status in specific tissues but it is still necessary to determine local T3 availability to the TRs, assuming that TRs require ligand occupancy in order to be effective (Power et al., 2001). T3 availability may also depend on the balance between deiodinases that both form and degrade T3 in each tissue. Pin-pointing TH action sites might therefore be furthered by studying tissue-specific mRNA deiodinase levels (Sambroni et al., 2001). During development also, there are changes in the levels of other hormones such as cortisol (de Jesus et al., 1990, 1991; Yamano et al., 1991) and it is unlikely that TH act alone. TH interact with cortisol (de Jesus et al., 1990; Brown and Kim, 1995; Sampath-Kumar et al., 1995) and prolactin (de Jesus et al., 1994) in fish development. Furthermore, retinoic acid affects fish development (Browman and Hawryshyn, 1994b; Miwa and Yamano, 1999; Suzuki et al., 2000; Haga et al., 2003) and could modify TH effects at the transcriptional level as indicated for zebrafish brain development (Essner et al., 1999).

Somewhat different is the metamorphosis of the filter-feeding lamprey larva (ammocoete) to its adult ectoparasitic form. This occurs after several years of larval life and uniquely involves dramatic changes in the mode of TH secretion from the endostylic to the follicular thyroid. Furthermore, in contrast to other vertebrates, completion of metamorphosis may depend

on a decrease in high pre-metamorphic TH levels, due in part to increased intestinal IRD activities (Eales et al., 2000).

Parr-smolt Transformation

Since early evidence of histological or radiochemical heightened thyroid activity of Atlantic salmon (Hoar, 1939) and Pacific salmon (Eales, 1963) at parr-smolt transformation (PST), numerous studies have established that at this salmonid stage, there is a marked increase in plasma T4 with little change in plasma T3 (reviewed by Dickhoff and Sullivan, 1987; Hoar, 1988; McCormick et al., 1998). Several studies suggest a natural sharp plasma T4 peak maintained for only a few days. However, under hatchery or laboratory conditions, protracted or less pronounced peaks can occur. This may reflect sampling from populations of fish of variable body size and physiologic state, which are asynchronous in their PST development. In nature, the timing of the plasma T4 surge appears finetuned and coordinated by environmental stimuli, including moon phase, local changes in water velocity, rainfall, turbidity, temperature and quality as well as a general susceptibility to disturbances (e.g., Grau et al., 1982; Hoffnagle and Fivizzani, 1990; McCormick et al., 1998; Specker et al., 2000).

Numerous TH roles have been proposed during PST (e.g., salinity preference, osmoregulation and seawater survival, olfactory imprinting, general activity, downstream migration, purine deposition and skin silvering, and changes in hemoglobin profiles and intermediary metabolism) but with little direct evidence for TH as the prime regulator (Hoar, 1988). Instead, TH may act permissively to enable the actions of other hormones, whose blood profiles, receptor abundance or production rates are also changing during PST and may contribute to smolt characteristics. These hormones include E2, testosterone, progesterone, cortisol, GH, IGF-I, somatostatin, prolactin, somatolactin and melatonin (e.g., Specker and Schreck, 1982; Miwa and Inui, 1985; Boeuf et al., 1989; Young et al., 1989; Sower et al., 1992; Yamada et al., 1993; Cowley et al., 1994; Nagae et al., 1994; Sakamoto et al., 1995; Porter et al., 1998; Sheridan et al., 1998). For example, TH appear to play a permissive role in changes in gill function and osmoregulation where the directive change depends on GH and where other hormones—including IGF-I, somatostatin and cortisol—may be involved (Leloup and Lebel, 1993; Boeuf et al., 1994; Trombetti et al., 1996; Shrimpton and McCormick, 1998, 2003).

A vital clue in understanding TH action during PST may lie in the low plasma T3 level accompanying the plasma T4 surge and which, in all likelihood, stems from low hepatic T4ORD activity (Whitesel, 1992; Specker et al., 2000). Thus for the T4 surge to be effective during PST, T4 itself must either act as the TR ligand or, through its heightened availability, drive T3 production in those extra-hepatic target tissues with the ability to form T3. If the latter, then target tissues can be identified not only from their TR abundance but also from their deiodination activities. On this basis, several potential target tissues exist in Atlantic salmon smolts (Eales et al., 1993; Morin et al., 1993; Sweeting et al., 1994). Of especial interest is the brain. In general, the entire fish brain has negligible T4ORD activity but appreciable T3IRD activity, suggesting that it does not generate T3 and regulates its T3 level by degradation of systemically-acquired T3 (Frith and Eales, 1996; Eales et al., 1997; Mol et al., 1997, 1998; Cyr et al., 1998; Fines et al., 1999; Adams et al., 2000; McLeese et al., 2000; Plate et al., 2002; Plohman et al., 2002b). Low brain T4ORD activity in trout is supported by the negligible level of type II deiodinase mRNA (Sambroni et al., 2001). However, during PST, significant T4ORD activity develops in Atlantic salmon whole brain (Eales et al., 1993; Morin et al., 1995; Specker et al., 2000), and, furthermore, can be synchronized with lowered T4IRD and T3IRD activities (Specker et al., 2000). The combined effect would increase T3 availability in the brain, implicating it, or a particular region, as a temporary target site during PST. Thus T3 acting on brain TRs (Kudo et al., 1994) could contribute to the altered neural circuitry and brain function reported during PST (Holmqvist et al., 1994; Parhar et al., 1994; Ebbesson et al., 1996a, b, 2003; Parhar and Iwata, 1996). In fact, experimental simulation of the smolt surge in plasma T4 in Atlantic salmon changed the brain monoaminergic activity specific to the olfactory system (Morin et al., 1997). Parenthetically, a *tonic* TH presence may also be needed for routine brain function in fish not undergoing PST. T3 levels and T3:T4 molar ratios are high in the brain (Specker et al., 1992; Plohman et al., 2002a); TRs occur in the brain (Bres and Eales, 1988; White et al., 1990; Dasmahapatra et al., 1991) and TH alter neural function (Hara et al., 1966; Ghosh et al., 1983; Ghosh and Medda, 1984; Medda and Ghosh, 1984; De et al., 1989b, 1992, 1993; Chaube and Joy, 2003), while over-expression of TR α1 during zebrafish embryogenesis disrupts hindbrain patterning (Essner et al., 1999).

TH may also affect sensory function during PST. In juvenile trout, T4 caused a change in UV photosensitivity characteristic of smolts (Browman

and Hawryshyn, 1992, 1994a). In coho parr, T3 increased the retinal rhodopsin:porphyropsin ratio and methimazole impaired this transition but the directional change was contrary to that expected at PST (Alexander et al., 1994, 1998). The salmonid retina also exhibits deiodination pathways (Orozco et al., 2000; Sambroni et al., 2001) that show regulation (Plate et al., 2002), further supporting the retina as a TH target tissue in salmonids. There has also been interest in TH involvement in home-stream odor imprinting at PST, with emphasis on a TH role to enhance the olfactory sensitivity (Hasler and Scholz, 1983; Morin et al., 1989; Dittman et al., 1994; Nevitt and Dittman, 1998). However, increased olfactory sensitivity (e.g., olfactory bulb electroencephalographic response to standardized stimuli = presumed index of imprinting) developed in late Atlantic salmon parr under a short photoperiod that did not induce the normal smolt surge in plasma T4 (Morin et al., 1996). Therefore, although for this species imprinting could depend on a certain TH tone, it does not appear to depend on a T4 surge. In fact, T4 treatment of late-parr to prematurely simulate the smolt T4 surge depressed olfactory sensitivity (Morin et al., 1995). Thus the T4 surge may terminate odor imprinting prior to migration and exposure to potentially confounding downstream odors. However, *tonic* TH levels could still be required for learning olfactory clues during migration as proposed in 'sequential' learning theories (McCormick et al., 1998).

TH-induced changes in brain and sensory function could contribute to smolt-specific behavioural changes (McCormick et al., 1998). Temporal correlations have been reported between changes in water conditions, plasma T4 levels and rheotropic behaviour of smolts and so downstream migration might be due to changes in plasma T4 levels (Grau et al., 1982; Youngson, 1984; Youngson and Simpson, 1984; Youngson et al., 1986, 1989; Fujioka et al., 1990; Specker et al., 2000; Iwata et al., 2003). However, experimentally altered thyroidal status does not necessarily change swimming behaviour in the expected manner (Birks et al., 1985; Youngson et al., 1989).

T4, under conditions of increased water velocity, may aid resistance to displacement but at steady velocities it may promote downstream swimming (Youngson et al., 1986). Ewing and Rogers (1998) found no differences in plasma T3 and T4 between migrant and non-migrant coho. The relationship between thyroidal status and swimming behavior appears complex and its investigation is further complicated by the rapidity with

which plasma T4 levels can alter in response to stressors and other stimuli (McCormick et al., 1998). TH may also promote migration in young salmonids by reducing aggression (Hutchinson and Iwata, 1998) and altering the phototactic behavior (Iwata et al., 1989). As with most aspects of PST, TH likely act with other hormones, such as cortisol, in developing PST behavioral changes (Iwata et al., 1990; Iwata, 1995).

In summary, major transitory changes in thyroidal status occur at PST, involving a large surge in plasma T4, little change in plasma T3, but tissue-specific changes in deiodination activities. This suggests that T4 drives T3 production in certain tissues. The brain and sensory system may be key target sites and so TH may contribute in a complex way to some of the observed behavioural changes at PST.

Reproduction

Reproductive strategies vary greatly among fish. Semelparous species reproduce once a year (or once in a lifetime), with major commitment of reserves to gamete production; iteroparous species reproduce several times and sometimes after short intervals; in further contrast, some ovoviviparous elasmobranchs have year-long gestations. Thus, reproductive biology, its underlying endocrinology and any TH involvement are likely to be highly species-specific. Furthermore, teasing out the reproductive roles of TH may be particularly challenging due to TH roles in other aspects of metabolism. Some earlier reviews (Eales, 1979; Leatherland, 1987a, 1994; Cyr and Eales, 1996) indicate that by several criteria thyroid status is increased during early oogenesis or spermatogenesis. Despite some recent negative findings (Moriyama et al., 1997; Lambert et al., 1999; Sohn et al., 1999; Jourdan et al., 2000; Norberg et al., 2004) several other recent research support this view (Mylonas et al., 1994, 1997, 1998; Björnsson et al., 1998; Persson et al., 1998; Poncin et al., 1999; Ruby and Eales, 1999; Pavlidis et al., 2000; Roy et al., 2000; Plohman et al., 2002a). Sambroni et al. (2001) reported deiodinase type II (T4ORD) mRNA in the ovary and testis with an increase at stages II and III of spermatogenesis. Kwon et al. (1999) observed correlations between thyroid status and various stages of vitellogenesis and development in a viviparous rockfish.

Several *in vivo* and *in vitro* experimental studies administering combinations of GTH forms and TH are consistent with the above correlations and suggest a TH permissive role to facilitate GTH action

(Cyr and Eales, 1996). More recent studies confirm this view (Tambets et al., 1997; Timmermans et al., 1998), but sustained T4 treatment alone did not improve reproductive performance of tilapia (Subburaju et al., 1999). TH (and particularly T3) are most effective at low GTH levels, suggesting a TH dependence in early ovarian development during commitment to reproduction (Cyr and Eales, 1996). Thyroid status generally correlates with energy balance (Eales, 1988; Dickhoff et al., 1989; Eales and MacLatchy, 1989) and so a threshold thyroid status may signal readiness to engage in energy-demanding gametogenesis (Eales, 1979). However, once committed to reproduction, TH may be unimportant for exogenous vitellogenesis in some semelparous species. Indeed plasma TH levels, and particularly T3, may fall dramatically in salmonids as gonadal growth, vitellogenesis and spermatogenesis proceed. This may reflect the decrease in metabolic reserves, accentuated in some cases by cessation of feeding, and in females it may also reflect the withdrawal of TH from the circulation and into the eggs (Tyler and Sumpter, 1996; Monteverdi and Di Giulo, 2000; Power et al., 2001). However, rising E2 levels also depress salmonid thyroid status Cyrand Eales, 1996; Wiens and Eales, 2005 and this may serve to suppress the somatic growth-promoting actions of TH that would otherwise compete with gonadal growth for finite energy reserves. In apparent contradiction, TH levels may increase during vitellogenesis, particularly in some iteroparous species. However, this is also consistent with thyroid status as an index of metabolic state with TH signaling adequate metabolic reserves for ongoing gonadal development.

In some species, including salmonids, there may be a much later permissive TH role in germinal-vesicle breakdown in females and spermiation in males. In some instances, plasma TH levels increase at this time and may be higher in males (Cyr and Eales, 1996). Datta et al. (1999, 2002) found T3 administration induced an ovarian protein that increased progesterone formation in an Indian perch by inducing 3β-hydroxysteroid dehydrogenase: D5-D4-isomerase activity. At this later stage of gametogenesis, TH levels may again reflect the metabolic state and energy reserves. Spawning and post-spawning activities may be energetically demanding, and although thyroid status in pre-spawners may be at a much lower level than at other times of the year, its relative change may still indicate metabolic readiness to complete spawning. Thus, thyroid status may also represent a critical permissive signal in timing the final gonadal development and spawning behaviour. Thyroid status is universally low after spawning but usually increases to pre-reproductive levels shortly

thereafter. This increase is often correlated with resumption of somatic growth and in rainbow trout is associated with an increase in plasma testosterone. Androgens can increase T3 production and plasma T3 (Cyr and Eales, 1996), while androgens and GH can act synergistically to promote somatic growth (Higgs et al., 1982).

In summary, there is a clear relationship between the gonadotropic and steroid hormones specifically regulating reproductive events and the TH generally reflecting metabolic state. By a complex interplay, these endocrine systems appear to regulate competing growth and reproductive processes. Through their largely permissive actions, the TH may ensure that reproduction proceeds under conditions that favor energetically its consummation.

THYROID DISRUPTING CHEMICALS

Both laboratory and field studies on almost 40 teleosts show that many diverse chemical contaminants (xenobiotics) modify thyroid end-points (reviewed by Leatherland, 1993, 1994, 2000; Kime, 1998; Eales et al., 1999; Rolland, 2000; Brown et al., 2004; Eales and Brown, 2005).They include aromatic hydrocarbons, planar halogenated aromatic hydrocarbons (dioxans, furans, coplanar PCBs), organochlorine, organophosphorus and carbamate pesticides, chlorinated paraffins, cyanide compounds, methyl bromide, phenol, ammonia, metals (aluminum, arsenic, cadmium, lead and mercury), low pH conditions, environmental steroids and a variety of pharmaceutical agents. Most data pertain to polychlorinated biphenyls (PCBs). There is current interest in E2-like contaminants but so far, few published studies exist on their effects on the fish thyroid.

Interpreting xenobiotic actions on the fish thyroid system is difficult (reviewed by Brown et al., 2004; Eales and Brown, 2005). Firstly, there are many thyroid end-points and hence many potential lesion sites within the thyroid hierarchy, ranging from I^- acquisition to TH binding to target receptors and to expression of physiologic responses. Precise mechanisms of action at these sites have not been established for most chemicals influencing the thyroid system. Secondly, the thyroid system responds *naturally* to many changes in environmental and physiologic states. Indeed, much fish thyroid literature (unrelated to ecotoxicants) documents thyroid responses to variables such as temperature, stressors, nutritional availability and sexual state. The thyroid system is also pivotal

in the total endocrine network. Consequently, a contaminant inducing a stress response, reducing appetite or even changing some other endocrine system could *secondarily, or indirectly*, modify the thyroid status. Most toxicant studies have not addressed this point. Thirdly, interpretation is complicated by the thyroid's capacity to compensate for deficiencies or excesses in TH availability (thyroid homeostasis). Thus, some changes in thyroid end-points may merely represent central or peripheral autoregulation without actual change in the thyroid status or disruption of TH-dependent physiologic processes. While a deficiency in TH production may ultimately lead to diminished thyroid status, its impact will depend on the size of thyroidal and extra-thyroidal TH stores, which are poorly known. Many studies may not have been sufficiently prolonged to reach the deficiency state. Therefore, although a chemical may change thyroid end-points, it is still uncertain if these responses represent merely *measures of exposure* or if they represent *measures of effect* accompanied by true changes in thyroid status. Finally, because TH in fish have mainly permissive roles, little progress has been made in identifying TH-specific physiologic end-points. However, aspects of early development, metamorphosis and PST show potential as model experimental systems.

CONCLUSIONS

There are >25,000 species of extant teleost and non-teleost fish. They differ greatly in their physiology, rendering it unlikely that a common thyroid model describing TH actions will accommodate all species. However, in the few species studied to date, there exist certain commonalities. They all secrete predominantly T4 from the thyroid, which is under central control through the BPT axis, resulting in plasma T4 levels that may show significant short-term fluctuations. T3, the presumed bioactive TH form, is generated from T4 by deiodination in extrathyroidal tissues by a suite of deiodinases. In some tissues (liver or kidney, depending on species), these deiodinases may be mainly responsible for regulating plasma T3 levels, which in contrast to T4 levels show considerable short-term stability. In other tissues, the deiodinases are likely responsible for regulating the local T3 availability. As expected, T3 has greater affinity than T4 for nuclear TRs, which are homologous with TRs of other vertebrates and there is every reason to believe that the TR/T3 complex regulates transcription and specific protein synthesis.

Difficulties in defining the modes of action and physiologic effects of TH in fish arise in part from the wide variety of factors that affect the thyroid system and the variety of processes that the TH, in turn, appear to influence. Experimental interpretation is further clouded by the thyroid capacity to compensate for natural and manipulated variations in TH availability. Thus, establishing TH roles is challenging, even in flawlessly designed experiments. In retrospect, there have been several oversights involving use of pharmacologic TH levels, use of inhibitors lacking rigorous controls and disregard for secondary changes induced in the thyroid hierarchy itself. Despite these limitations and challenges, over half a century of research strongly suggests TH roles in somatic growth, early development and metamorphosis, parr-smolt transformation and reproduction. Knowledge of precise actions of TH in these processes remain elusive due to the permissive roles that TH tend to play in fish. Often, other hormones appear to provide the definitive directive signals but their efficacies appear to depend on TH. Thus, a critical T3 tone may give the 'green light' for these energy-demanding processes. Consistent with this, thyroid status itself responds to a broad range of environmental variables which generally suggest activation in anabolic states and inhibition in stressful/catabolic states. Nevertheless, for certain aspects of metamorphosis and PST more definitive and directive TH roles may have evolved. In light of the above it is, therefore, not surprising that although numerous environmental chemical (toxicants) can alter thyroid end-points, the actual impacts of these toxicants on thyroid status and thyroid-regulated processes are still poorly understood.

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CHAPTER

24

The Impact of Environmental Hormonally Active Substances on the Endocrine and Immune Systems of Fish

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ABSTRACT

A variety of environmental substances, designated as endocrine disrupting compounds (EDCs), are able to interfere with the endocrine system of exposed organisms by mimicking hormone action or by disturbing hormone synthesis and metabolism. To date, most attention in research on endocrine disruption in fish has been given to the action of estrogen-active compounds acting through binding to the estrogen receptor. However, mechanisms other than receptor binding and hormone systems other than the estrogen system can be impacted as well. This chapter considers three potential targets of EDCs:

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sexual differentiation of the gonads; the IGF-I hormone system, and the immune system. EDCs have been found to affect gonadal differentiation of fish in various ways. In order to go beyond descriptive, phenological studies and to classify and understand the various response patterns, it is essential to include mechanical and physiological information. To this end, different patterns and timing of sexual development of fish (e.g., differentiated versus undifferentiated gonochorists), the different organs involved in sexual development (e.g., brain, pituitary and gonads), as well as different modes of EDC action (e.g., effects mediated through ligand binding versus effects mediated through inhibition of steroidogenesis) have to be taken into account. To date, the available findings on the disrupting effects of environmental hormone-active substances on the IGF-I and the immune system are still rather preliminary. In particular, the mechanisms by which EDCs may modulate growth and immune responses are not known, and there is no understanding as to how EDCs may interfere with the crosstalk between different endocrine systems. It will be a major challenge of future research on EDCs to better address this complexity of the endocrine system and its consequences for EDC effects. Further, the possible impact of EDCs on the IGF-I and the immune system indicates that population-level consequences of EDC exposure may not only arise through disruption of development and reproduction, but also by compromising growth and disease resistance.

Key Words: Sexual differentiation; Endocrine disruption; Estrogens; Growth; IGF-I; Immune system.

INTRODUCTION

A variety of environmental substances has been shown to be able to interfere with the endocrine system of exposed organisms either by mimicking hormone action or by disturbing hormone synthesis and metabolism. These substances are designated as endocrine-disrupting compounds (EDCs), as endocrine modulators or endocrine-active compounds. Through their effects on metabolism, distribution, or function of endogenous hormones, EDCs have the potential to disrupt hormone-controlled physiological processes such as development, growth, stress responses, sexual differentiation or reproduction. Examples of environmental EDCs include synthetic estrogens (e.g., from contraceptives), natural estrogens (e.g., from human excretion), phytoestrogens (e.g., from soy or wood-processing), chlorinated pesticides such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), polybrominated flame retardants (PBDEs), and

industrial chemicals such as alkylphenols or phtalates. Organisms have evolved sensitivity to endogenous and exogenous chemical signals as a means to adaptively respond to physical or biological stimuli in the environment and to maintain internal homeostasis. However, this sensitivity simultaneously makes organisms vulnerable to inadvertent signals in their environment (Cheek et al., 1998).

The endocrine disruption hypothesis claims that synthetic as well as naturally occurring chemical substances in the environment disrupt the normal functions of the endocrine system and its hormones in humans and wildlife. This hypothesis has received significant scientific and public attention during the last 20 years. (Colborn and Clement, 1992; McLachlan, 2001; Matthiessen, 2003). A number of adverse alterations observed in wildlife, e.g., the disturbed sexual development in birds (Fry and Toone, 1981) or the occurrence of intersex in wild fish (Jobling et al., 1998), has been thought to be caused by exposure to EDCs. Also for man, exposure to EDCs has been discussed as a possible cause of changes in sperm quality and quantity (e.g., Sharpe and Skakkebaek, 1993) or as cause of increases in some types of breast, prostate and testicular cancer (Colborn et al., 1993; Safe and Zacharewski, 1997).

The aquatic environment is a sink for chemical substances and, therefore, aquatic animals are particularly endangered by the action of EDCs (Tyler et al., 1998). In fact, perhaps the clearest evidence for endocrine disruption resulting from a chemical pollutant is the induction of male sexual organs in female marine snails (Bright and Ellis, 1990; Oehlmann and Schulte-Oehlmann, 2003). This condition, called 'imposex', leads to infertility and has caused population declines of snails in many coastal areas. The cause of this phenomenon is attributed to tributyltin, a pesticide contained in marine antifouling paints. Also for teleost fish, observations such as vitellogenin (VTG) appearance in male fish (Purdom et al., 1994; Allen et al., 1999) or gonadal and hormonal alterations (Jobling et al., 1998; Munkittrick et al., 1998; Bortone and Cody, 1999) have attracted attention to the contamination of the aquatic environment by EDCs and their potential impact on the health of wild fish populations (Jobling and Tyler, 2003a). The original work on freshwater fish, conducted in the UK, established that treated sewage effluents were estrogenic, inducing VTG in male fish (Purdom et al., 1994). The estrogen-active substances contained in the effluents comprise a major part of natural and synthetic hormones as also synthetic substances such

as alkylphenols (Desbrow et al., 1998). In addition to VTG induction, exposure to treated sewage effluents has also been associated with pathological alterations of gonad morphology such as formation of intersex gonads, i.e., feminization of reproductive ducts and/or occurrence of oocytes in predominantly testicular tissue. Intersex as a consequence of exposure to estrogenic effluents has been most intensively studied in the roach, *Rutilus rutilus* (Jobling et al., 1998), and evidence has been presented that intersex roach are compromised in their reproductive capacity (Jobling et al., 2002).

To date, most attention in research on endocrine disruption in fish has been given to the action of estrogen-active compounds, i.e., compounds that exert their activity through ligand binding to the estrogen receptor. However, other hormonal systems can be impacted as well, e.g., the thyroid system (see: Eales, this volume), the hypophysis-interrenal axis (Hontela, 1998) or neuroendocrine systems (Jalabert et al., 2000). The present report will focus on three potential target systems of endocrine-active environmental chemicals—sexual differentiation of the gonads, growth regulation, and the immune system.

SEXUAL DIFFERENTIATION OF THE GONADS OF TELEOST FISH AND THE IMPACT OF EDCS

Two processes have to be distinguished in sexual development, i.e., genetic sex determination and phenotypic sex differentiation. In mammals, for instance, the initial decision to direct the bipotential genital ridge into either the male or female pathway of development is under control of a number of genes (Parker et al., 1999; Scherer, 1999; Hughes et al., 2003). One of the more significant genetic regulators of sex in mammals is *SRY*, located on the short arm of the Y chromosome (Koopman et al., 2001; Clarkson and Harley, 2002). This gene has a small time window of male-specific expression in the genital ridge of the fetus, and programs the originally bipotential genital ridge to differentiate into testes. If SRY expression during that critical window is absent, the genital ridge develops into an ovary. Corresponding knowledge on the nature of the sex-determining genes in fish is not available to date, although the presence of various genes known to be involved in mammalian sex determination, e.g., *DMRT1/DMY* and *SOX9* genes, has been recently demonstrated (Marchand et al., 2000; Lu et al., 2003; Scholz et al., 2003; Volff et al., 2003).

Sexual differentiation relates to the events that allow expression of genetic sex into the appropriate phenotypic sex. The ontogenetic process of sex differentiation is coordinated by a cascade of endocrine factors, particularly by hormones of the hypothalamus-pituitary-gonad (HPG) axis, as well as by paracrine factors, for instance, growth factors (see below). Hormones direct the differentiation of cells and tissues (such as the gonads) into organs with proper structure and the capability of responding correctly to external and internal cues. Such effects, where exposure to hormones early in life induces permanent alterations becoming apparent in later life are called 'organizational' effects, contrary to 'activational' effects, which represent transitory responses of cells and tissues to exogenous or endogenous stimuli (Guillette et al., 1995). The organizational action of hormones on the differentiation processes is dependent on being released at specific times in development within a specific dose range (e.g., Iguchi, 1992; vom Saal et al., 1997; Bigsby et al., 1999; McLachlan, 2001).

Sex steroids have an organising effect on the phenotypic expression of male or female genitalia in vertebrates. In the male mouse, Leydig cells start to synthesize testosterone during the early fetal stage. Testosterone triggers an orderly sequence of differentiation events, from the formation and stabilisation of the Wolffian duct to testes descent into the scrotum (Hughes et al., 2003). If alterations of sex steroid homeostasis occur during this developmental stage, even if the changes are small and transitory, they may result in persisting alterations of phenotypic sexual traits and reproductive capacity (vom Saal et al., 1997; McLachlan, 2001).

Morphological Differentiation of the Gonads

Fish gonads are composed of somatic and germ cells. The somatic cells are believed to derive from cells of the peritoneal wall and thus to have a monistic origin, what is in contrast to most other vertebrates, where the stromal cells in the developing gonad are of dual origin, the peritoneal wall and the mesonephric blastema (Nakamura et al., 1998). Prior to sexual differentiation, the germ cells are called primordial germ cells (PGCs). PGCs are set aside from somatic cell lineages during early embryonic development. The mechanisms controlling PGC specification in fish have not been fully understood to date, but recent evidence indicates that it is dependent on the asymmetrical distribution of maternally inherited

material in the developing embryo (Yoshizaki et al., 2003). The investigation of PGC development during the embryonic stages has been facilitated when it was shown that the *vasa* gene, which is a marker for the germ cell lineage in mammals, can be used as marker in fish as well (Olson et al., 1997; Yoon et al., 1997). The PGCs emerge in extragonadal areas and migrate to the germinal ridges where they coalesce with somatic elements to form the early, still undifferentiated gonads (Patiño and Takashima, 1995; Braat et al., 1999; Devlin and Nagahama, 2002; Yoshizaki et al., 2003). Subsequently, the gonads undergo a period of slow growth, with proliferation of both somatic and germ cells. This period can last for days, months or even years, depending on the species (Piferrer, 2001; Devlin and Nagahama, 2002). The early gonads are usually found at the dorsal wall of the coelomic cavity. In zebrafish for instance, they are located close to the distal end of the swimbladder (Takahashi, 1977; Maack and Segner, 2003). The germ cells can be recognized histologically by their large size, low nucleocytoplasmic ratio, clear nuclear borders and granular nuclear chromatin (Nagai et al., 2001). At that early stage of gonad development, the PGCs are still bipotential, i.e., they possess the ability to become either oogonia or spermatogonia, and do not yet express male-or female-specific characteristics, as elegantly shown by Parmentier and Timmermans (1985). In morphologically undifferentiated gonads of carp, PGCs of all individuals, irrespective of genetic sex, displayed sperm-specific antigenic determinants on their surfaces. When the early, undifferentiated gonads developed into testes, the germ cells continued to express sperm-specific antigens throughout all stages of sperm maturation from spermatogonia up to spermatozoa. In contrast, gonads that differentiated into ovaries, maintained the expression of sperm-specific antigens on the surface of germ cells only until the oogonia and early prophase stages but these antigenic determinants disappeared on follicular oocytes.

Transition of the early, morphologically indifferent gonads into gonads with sexually distinct morphology can follow different patterns (Yamamoto, 1969; Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). Many teleost species are gonochorists, i.e., individuals develop as either males or females and retain the same sex throughout life. Two types of gonochorism have been discerned. In differentiated gonochorists such as coho salmon (*Oncorhynchus kisutch*), carp (*Cyprinus carpio*), and sea bass (*Dicentrachus labrax*), ovarian and testicular differentiation proceed directly from the undifferentiated gonad, although

the two events may take place not simultaneously (usually, testicular morphological differentiation occurs later than ovarian differentiation) (Fig. 24.1). Undifferentiated gonochorists (transient or non-functional hermaphrodites) pass during development through a non-functional female phase when the gonads of all individuals, irrespective of the genetic sex, display primary stage oocytes. After this non-functional all-female stage with an immature ovary, in approximately 50% of the individuals, the ovarian tissue degenerates and resolves into normal, functional testes, while the other half of the population proceeds to develop mature, functional ovaries. This transition process can be accompanied by the invasion of gonad tissue of somatic cells and by transient presence of intersexual gonads, i.e., gonads that contain both male and female germ cells. An example of this type of development is the zebrafish (*Danio rerio*) (Takahashi, 1977; Uchida et al., 2002; Maack and Segner, 2003), although recent findings indicate that zebrafish possibly can develop both as differentiated and undifferentiated gonochorist (Hsiao and Tsai, 2003).

Apart from undifferentiated gonochorism or non-functional hermaphroditism, a number of fish species develop as functional hermaphrodites. This developmental pattern includes synchronous (also called simultaneous) as well as sequential hermaphrodites, i.e., protandric species which mature first as males and turn into functional females, or

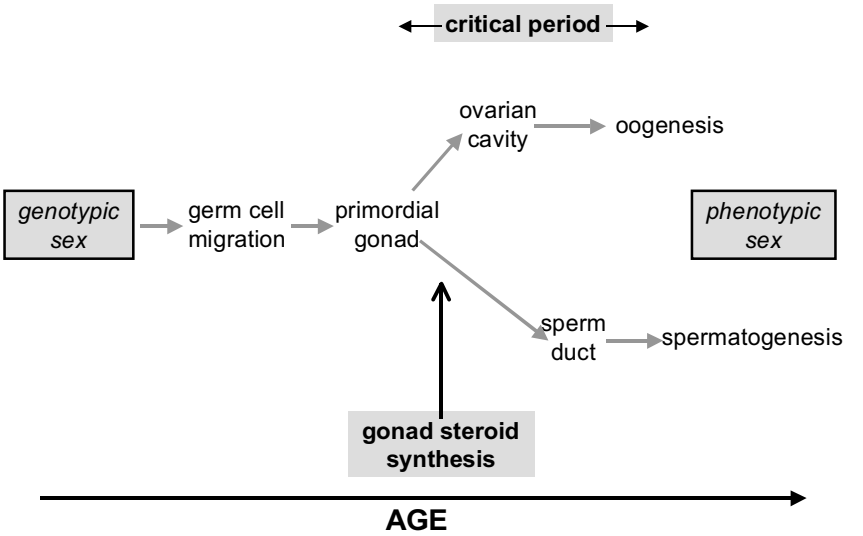


Fig. 24.1 Scheme of gonad development in a prototypic differentiated gonochoristic species.

protogynic species which develop first as functional females before they change into males. An example of a protandric fish species is seabream, *Sparus aurata*, while examples of protogynic species are found among coral reef fishes such as Serranidae. Synchronous hermaphrodites produce both male and female gametes at the same time, of which some species are capable of alternating between sperm and egg delivery, while species such as *Rivulus marmoratus* are able of internal self fertilisation. The different forms of hermaphroditism have now been observed in at least 25 families of fish, indicating the widespread nature of these modes of sexual differentiation (see: Devlin and Nagahama, 2002).

The developmental period when sexual differentiation of the gonad takes place varies greatly among teleost species (Piferrer, 2001). Early signs of the onset of ovarian differentiation are the entry of germ cells into meiosis and alterations of somatic cell arrangement that eventually lead to formation of the ovarian cavity (Nakamura et al., 1998; Strüssmann and Nakamura, 2002). This cavity is formed by proliferation of somatic cells. In males, an early sign of testicular differentiation is the appearance of the efferent duct as a slit-like space in the stromal tissue; further indications are intensive germ cell mitosis and formation of germ cell cysts (Strüssmann and Nakamura, 2002). As mentioned above, testis development of gonochoristic species usually occurs later than ovarian differentiation; for instance, in Nile tilapia, morphological gonad differentiation was observed 35 days post hatch (dph) in female fish, while it took 70 dph in male fish (Strüssmann and Nakamura, 2002).

Following morphological differentiation of the gonads, the next stages in sexual development are puberty (Schulz and Goos, 1999; Okuzawa, 2002), and, finally, the mature, reproductively active stage.

Hormonal Regulation of Gonadal Sex Differentiation

Sexual differentiation of developing gonads in fish is under the control of hormones of the hypothalamo-pituitary-gonadal (HPG) axis. In particular, sex steroids have the ability to act as organizers of phenotypic sexual differentiation of the gonads (Blazquez et al., 1998; Jalabert et al., 2000; Baroiller and D'Cotta, 2001; Piferrer, 2001; Devlin, and Nagahama 2002). Although steroid biosynthesis may not represent the initial event, sex steroids play a critical role in early differentiation of the gonads into the two sexual types, and probably also for the subsequent maintenance of the differentiated types (Devlin and Nagahama, 2002). The hypothesis on sex steroids as organisers of gonadal sex differentiation in fish has been put

forward by Yamamoto (1969) who found in medaka that, irrespective of genetic sex determination, estrogen treatment leads to differentiation into the female phenotype, and androgen treatment to the male phenotype. Since then, numerous studies including recent molecular work (see Baroiller and, D'Cotta, 2001; Devlin and Nagahama, 2002) have corroborated and extended the original hypothesis of Yamamoto (1969), although controversial observations do exist (e.g., Kawahara and Yamashita, 2000; Strüssmann and Nakamura, 2002). In this context, Strüssmann and Nakamura (2002) distinguish genera and families which appear to have a rather stable genotypic sex determination (Poecilidae, Adrianichthyidae) from those which show pronounced plasticity of phenotypic sex differentiation (Atherinidae, Salmonidae, Cichlidae, Paralichthyidae, possibly Moronidae).

If sex steroids function as morphogenic factor in gonadal differentiation in teleost fish, this implicates that sex-specific synthesis of steroid hormones has to start before morphological differentiation of the gonad tissue. This, in fact, has been demonstrated for several species. Electron microscopical investigations showed that cells with the ultrastructural characteristics of steroid-producing cells, such as the follicle cells in the ovary and the Leydig cells in the testis, are present already before or at the onset of gonad morphological differentiation (e.g., Nakamura and Nagahama, 1989). Further evidence of active steroid biosynthesis previous to gonadal morphological differentiation is derived from enzyme histochemical and immunohistochemical studies. Histologically, indifferent gonads of rainbow trout (*Oncorhynchus mykiss*) were shown to contain several steroidogenic enzymes including 3β-hydroxysteroid dehydrogenase (3HSD) and 11β-hydroxylase (P450c11) (van den Hurk et al., 1982; Kobayashi et al., 1998). In tilapia, major steroidogenic enzymes such as 3HSD and cholesterol side-chain cleavage cytochrome P450 (P450scc) could be detected immunohistochemically in the gonad anlage 10-15 days before morphological sex differentiation (Nagahama, 2000). The presence of steroid-synthesising enzymes in the gonads prior to and at the onset of morphological differentiation has also been demonstrated by molecular biological techniques (Guiguen et al., 1999; Govoroun et al., 2001). Finally, the importance of sex steroids in teleostean gonad differentiation is indicated by pharmacological experiments using inhibitors of steroidogenic enzymes. For instance, Piferrer et al. (1994) demonstrated that treatment of chinook salmon with an aromatase inhibitor during the period of sex differentiation caused

genetically female fish to develop as functional phenotypic males, and this sex reversion was irreversible. Congruent findings have been reported by a number of other authors (e.g., Guiguen et al., 1999; Kwon et al., 2000). Together, these findings support the hypothesis that sex steroids are organisers of sex differentiation in fish. Gonadal steroid synthesis appears to be sex-specific right from the onset of steroidogenic enzyme expression: for instance, in prospective ovaries of developing tilapia and rainbow trout, the enzyme aromatase (CYP19), which converts androgens into estrogens, was found at high levels, while in prospective testes, CYP19 was absent or expressed at low levels (Kobayashi et al., 1998; Nakamura et al., 1998; Guiguen et al., 1999). As recently discussed by Nakamura et al. (2003) it appears to be not the androgen level which promotes testicular development but low levels of estrogens which are below the threshold levels required for ovarian differentiation.

The ontogenetic period when endogenous synthesis of sex steroids increases and gonad development is directed into the female or male direction, is called the labile or critical period of sexual differentiation (Piferrer, 2001). During that period, the sexually still undifferentiated gonads are susceptible to the organizational effects of steroids (Nakamura and Takahashi, 1973; Hackmann and Reinboth, 1974; Blazquez et al., 1998; Piferrer, 2001). In gonochoristic fish, the critical period covers the developmental window prior to or concomitant with the histological differentiation of the indifferent gonad into either ovary or testis. The chronological timing of this period can vary strongly among fish species, from the embyro/hatching stage (e.g., in salmonids) until the juvenile stage (Piferrer, 2001). Gonadal sensitivity to the organizing effect of sex steroids is believed to be restricted to the critical period of development, whereas in adult (gonochoristic) fish, sex reversal by steroid treatment may be no longer possible due to the loss of bipotentiality of the germ cells. However, this hypothesis has been challenged recently.

The aforementioned studies focused on gonochoristic species, but steroids also act as organizers of gonadal sex differentiation in hermaphroditic fish species (Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002). For instance, the natural and controlled sex change in the Protandrous black porgy, *Acanthopagrus schlegeli* has been shown to depend on the development of the endogenous estrogen system (Lee et al., 2001). In the protogynic hermaphrodite *Thalassoma duperrey*, changes in steroidogenic enzyme expression and plasma steroid levels correlate with sex change (Morrey et al., 1998). Preceding and accompanying to

ovarian degeneration, a decline of gonadal CYP19 expression and of serum 17β-estradiol (E2) concentrations to non-detectable levels takes place. In parallel, gonadal 11β-hydroxylase (11-OH) mRNA is upregulated and increasing serum levels of 11-ketotestosterone can be found. Similar observations have been reported for other hermaphrodites, both protogynic and protandric ones (e.g., Kroon and Liley, 2000; Ohta et al., 2003). It is not clear yet whether the natural sex reversal of sequential hermaphrodites relies on remaining bipotential germ cells in the differentiated gonad, or if it is based on the reversal of already differentiated germ cells (Nakamura et al., 2003).

The factors triggering onset of sex-specific steroidogenesis in the early, still indifferent gonads of fish remain unknown to date. Recently, it has been shown that homologues of the fushi tarazu factor-1 (FTZ-F1), a member of the nuclear receptor superfamily, occur in early life stages of zebrafish, *Danio rerio* (von Hofsten et al., 2001). In mammals, two FTZ-F1 homologues have been cloned, i.e., the SF-1/Ad4BP (steroidogenic factor-1/adrenal 4 binding protein) gene and the LRH/FTF (liver receptor hormone/α-fetoprotein) gene. The SF-1/Ad4BP genes are important regulators of steroid biosynthesis and closely connected to sex determination, since they regulate transcription of the Müllerian Inhibiting Factor. Whether the zebrafish SF-1/FTF-homologue fulfills a similar function in sexual development of zebrafish remains to be clarified. Further, the role of the neuroendocrine system in controlling onset and direction of gonadal steroidogenesis must not be overlooked, although current understanding of the hypothalamic-pituitary regulation of gonad differention in fish is rather fragmentary. Better understanding is also needed for the role of the neuroendocrine system in regulating gonad differentiation. In adult fish, gonad steroid synthesis depends on the neuroendocrine hypothalamic-pituitary system (Grandi et al., 2003), and there exists evidence that this is the case with developing fish as well. For several fish species, it could be shown that the ontogeny of gonadotropic factors in hypothalamus and pituitary precedes gonad sex differentiation (Parhar, 1997; Devlin and Nagahama, 2002: Strüssmann and Nakamura, 2002).

Gonad Differentiation of Teleost Fish as Target of EDCs

Field studies on aquatic wildlife have provided evidence for an association between exposure to hormonally active chemicals and disturbances of gonad differentiation. In fish, the widespread occurrence of intersex, i.e.,

simultaneous presence of both male and female gonadal characteristics in the same fish, has been correlated to exposure to estrogen-active effluents from sewage treatment plants in a number of freshwater fish species such as roach, *Rutilus rutilus* (Jobling et al., 1998), bream, *Abramis abramis* (Hecker et al., 2002), gudgeon, *Gobio gobio* (van Aerle et al., 2001) or the stickleback, *Gasterosteus aculeatus* (Gercken and Sordyl, 2002). These gonadal alterations probably result at least partly from developmental exposure of fish, although this has been not unequivocally proven as yet. In birds, adverse alterations of gonad development were associated with exposure to chlorinated insectides, particularly DDT and its metabolites (Palmiter and Mulvihill, 1978; Fry and Toone, 1981). In red-eared slider turtles, it has been shown that developmental exposure to certain congeners of PCBs induces gonad sex reversal (Crews et al., 1995). Together, these observations point to sexual differention of the gonads as a critical target of EDCs in aquatic wildlife.

It is important to distinguish between EDC effects on developing and on already differentiated or mature gonads. While exposure during differentiation of the gonads has the potential to irreversibly alter gonad phenotype, e.g., reversing a genetically male gonad to a phenotypically female gonad (see below: critical period concept), exposure to EDCs of adult fish with an already differentiated gonad possibly results in an only transitory alteration of the differentiated gonad's structures and functions. For instance, when fathead minnow are exposed to estrogens from hatching onwards, sex reversal takes place, i.e., genetic males develop normal ovaries, whereas estrogen exposure during adult life does not induce sex reversal but pathological alterations of the gonads (Miles-Richardson et al., 1999; Länge et al., 2001). In medaka, the ability of EDCs to induce ovotestis was different in adult and developing fish (Gray et al., 1999). Zebrafish treated with the aromatase inhibitor, fadrozole, during the period of gonad differentiation show 100% masculinisation, i.e. individuals which under control conditions would have developed ovaries now display (morphologically normal) testes (Fig. 24.2a). In contrast, treatment of adult zebrafish with fadrozole does not induce masculinisation but induces pathological alterations of the ovaries (Fig. 24.2b), while testicular morphology remains unaffected (Segner, unpublished).

Generally, timing of exposure is a crucial factor when it comes to the investigation of EDC effects on gonad differentiation in developing fish. As laid out before, sex steroids have organisational effects on phenotypic

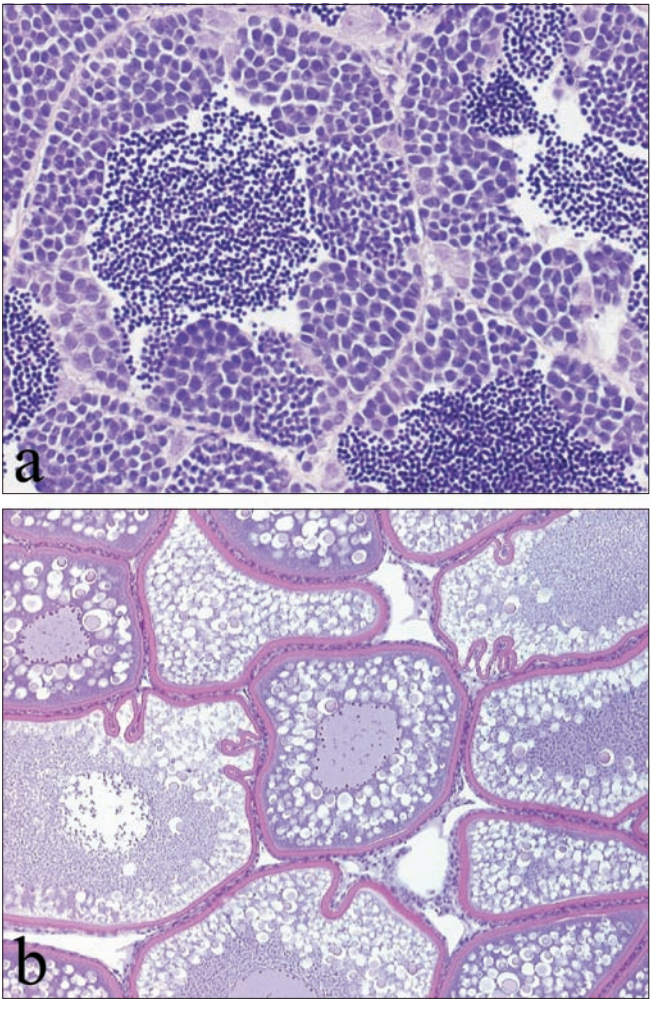


Fig. 24.2 **(a)** Gonad morphology of adult zebrafish treated during the juvenile stage with the aromatase inhibitor fadrozole. Juvenile fish were fed during gonad differentiation with a fadrozole-containing diet (500 µg fadrozole/mg food) and subsequently reared under control conditions until maturity; then histological examination of the gonads was performed. One hundred percent of fadrozole-treated fish showed gonads with testicular morphology; testes were fully differentiated and displayed no pathological lesions (for details: see Fenske and Segner, 2004). **(b)** Gonad morphology of adult zebrafish treated during adult stage with fadrozole. Adult fish were fed during a 3-week-period with a fadrozole-containing diet (500 µg fadrozole/mg food), and then examined histologically. Testicular morphology was not different from control fish, but in the oocytes, pathological changes such as invaginations of the oocyte membrane were observed. (Segner, unpublished).

sexual differentiation of fish gonads, i.e., the differentiation of the bipotential germ cells into spermatogonia or oogonia can be controlled by the relative amounts of estrogens and androgens to which the developing gonads are exposed to during the critical period. Hence, in many fish species, exposure to EDCs affecting endogenous steroid balance at the right time and dose during ontogeny can result in phenotypic feminization or masculinization. An excellent example of how EDC effects on developing fish change in relation to the time point when the substances are administered is provided by the work of van Aerle and colleagues (2002, 2004). These studies further indicated that not only the time point when the EDC effect is administered but that also the time point when the effect is measured can be critical, since treatment-induced alterations such as morphological sex reversal or ovo-testis may be reversible (see also Seki et al., 2003; Maack and Segner, 2004; Fenske et al., 2005).

Exogenous induction of sex reversal in gonochoristic fish by applying exogenous steroids during the critical period of development leads to fully functional gonads. In aquaculture, the possibility to manipulate sexual differentiation of fish is practically utilized to produce monosex populations (Piferrer, 2001). For this intended sex reversal, usually high, supra-physiological concentrations of steroids are applied. This is different to the exposure situation of feral fish, since the concentrations of hormonally active contaminants in the aquatic environment are usually low. Therefore, the question is whether exposure of developing fish to environmentally relevant concentrations of EDCs is able to result in persisting alterations of gonad sexual differentiation and reproductive capabilities. The mechanisms of actions of exogenous EDCs on gonad differentiation in fish are not sufficiently understood, but in principal, they could be mediated through one of the following modes of actions:

- interference with estrogen signaling pathways
- interference with androgen signaling pathways
- interference with endogenous steroid metabolism

It is important to realize that EDCs can modulate gonad development and physiology not only by one mode of action but in various ways. For instance, ER and AR are present in both ovaries and testes (e.g., Bouma and Nagler, 2001), thereby providing a basis for the effects of exogenous estrogen- or androgen-active compounds in both sexes. Further, exposure to steroideal compounds possibly does not only affect the receptor pathways but may simultaneously stimulate or inhibit the activities of steroidogenic enzymes via substrate feedback mechanisms (Chang et al.,

1999; Kitano et al., 2000). Finally, environmental EDCs may disrupt gonad physiology not only directly by modifying gonadal steroidogenic activity or by influencing ER- or AR-dependent processes in the gonads, but also indirectly via an impact on the neuroendocrine system. For instance, while the gonadal aromatase isoform in zebrafish is not responsive to estrogens, the brain aromatase isoform is responsive (Kishida and Callard, 2001; Menuet et al., 2003; Kazeto et al., 2004), so that the effect of environmental estrogens on zebrafish gonads may occur through alterations of cerebral steroid metabolism. EDCs can also alter neurotransmitter concentrations and, thereby, gonadotropin synthesis of fish (Khan and Thomas, 1997).

As endpoints to assess an EDC effect on gonad development, mainly gonad morphology and changes of phenotypic sex ratio are used. With species such as zebrafish where genetic sex determination is not understood, deviation from sex ratios in control populations serve as basis for identifying EDC effects. The evaluation of treatment effects is facilitated when monosex populations are available. For species such as medaka, where genetic sex markers do exist, direct comparison of phenotypic and genotypic sex is possible.

Effect of estrogen receptor-binding substances on gonad differentiation of teleost fish

The by-far most intensively studied target of EDC action is the estrogen receptor (ER). In mammals, there are two subtypes, ERα and ERβ, and also in various fish species, ERα and ERβ have been identified (Legler et al., 2000; Kishida and Callard, 2001; Menuet et al., 2002). Recently, an ERγ or ERβ2 has been characterized in some fish species, including Atlantic croaker (*Micropogonias undulatus*) (Hawkins et al., 2000) and zebrafish (Kishida and Callard, 2001; Menuet et al., 2002). Phylogenetic analysis indicated that ERβ2 arose by gene duplication of the ERβ gene early in teleost evolution. Upon ligand binding, the ER is conformationally changed, dimerised and interacts as receptor-ligand complex with estrogen responsive elements in the DNA. Subsequently, the DNA-bound receptors interact with the transcription machinery directly or via coactivator proteins (Hall et al., 2001). Natural and synthetic estrogens and anti-estrogens as well as non-steroidal estrogen mimics can act as ligands of the ERs and activate the ER-signaling pathway (Madigou et al., 2001; Katzenellenbogen and Muhtyala, 2003). Environmental ER ligands

include substances such as the synthetic estrogen ethinylestradiol (EE2) which is released into the aquatic environment via effluents of sewage treatment plants, but they also include non-steroideal xenobiotics such as nonylphenol, octylphenol, bisphenol A or certain chlorinated aromatic substances (Katzenellenbogen and Muthyala, 2003). The affinity of the non-steroideal estrogen mimics to ER is usually 2-3 orders of magnitude lower than that of the natural ligand E2. Environmental substances may also affect the ER pathway indirectly. This has been shown for dioxin-like compounds which bind to the arylhydrocarbon receptor. Ligand activation of this receptor by dioxin-like compounds leads to suppression of ER-mediated responses, and thereby exerts anti-estrogenic activity (Safe, 1995; Navas and Segner, 2001; Ohtake et al., 2003).

The impact of estrogen-active EDCs on gonad sexual differentiation during ontogeny has been studied for several fish species developing as differentiated gonochorists. Gimeno et al. (1998) exposed monosex male carp to the weak estrogen, 4-tert-pentylphenol (36, 90 or 256 μg TPP/L) and to E2 as positive control (9 or 23 μg E2/L) during the period of gonad sexual differentiation, starting at 50 dph. While TPP did not affect fish growth, in either, the low and the high E2 treatment, fish grew 25-45% less than the controls. After 20 days of exposure (i.e., 70 dph), the gonads of both, control and TPP-exposed fish were at an undifferentiated stage with PGCs and showed the typical golf club shape (see: Parmentier and Timmermans, 1985). In contrast, all fish exposed to 9 or 23 μg E2/L had developed oviducts, indicating the onset of phenotypic feminization of the genetic males. In TPP-treated fish, oviduct formation was first observed after 30 days of exposure, while gonads of control fish were still undifferentiated at that time. After 90 days of treatment, 100% of the E2-exposed carp possessed oviducts, while in the TPP groups, the percentage of fish showing an oviduct was concentration-dependent: in the groups exposed to 90 or 256 μg TPP/L, all individuals had developed an oviduct, but in the 36 μg TPP/L group, only 50% of the fish possessed an oviduct. Changes in the reproductive duct were accompanied by corresponding responses of the germ cells. In control fish, PGC proliferation started around 50 dph, and spermatogenesis was observed around 120 dph. In the gonads of the E2-treated genetic males, the first signs of oogenesis were observed after 50 days of exposure (i.e., 100 dph), for both E2 concentrations and, subsequently, oocyte development proceeded as in a normal ovary. With TPP, those fish which had developed oviducts

initiated spermatogenesis, although at a much lower rate than in controls; sometimes intersex, i.e., oocytes in spermatogenic tissue, was observed. The results from this study correspond to findings from experiments on intended sex reversal in aquaculture: strong estrogen treatment leads to full phenotypic sex reversal, i.e., the morphology of the reversed ovaries in the genetic males can not be distinguished from ovaries as present in genetic females, while weak estrogen treatment at low doses results in only a partial phenotypic reversal of gonad morphology (cf. Piferrer, 2001; Devlin and Nagahama, 2002). Since the TPP concentrations used in the work of Gimeno et al. (1998) were in an environmentally realistic range, the results would implicate that environmentally relevant concentrations of (xeno)estrogens have the potential to disrupt gonad differentiation in developing carp, be it by phenotypic sex reversal or by adverse alterations of gonad morphology and/or function.

In gonochoristic salmonids, high dose treatment with sex steroids during the critical period of gonad differentiation can induce complete and functional sex reversal (for review see Piferrer, 2001; Devlin and Nagahama, 2002). Sexual differentiation in salmonids occurs between hatching and yolk sac absorption and, during this period, the gonads are sensitive to hormonal treatment (Krisfalusi and Nagler, 2000). Carlson et al. (2000) microinjected eggs of rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) with low doses of endocrine-active DDE isomers or octylphenol, and this resulted in exposure of the developing fish during the period of gonad differentiation. The treatment failed to alter sexual development and the authors concluded that bioaccumulated residues of EDCs usually would be insufficient to disrupt gonad development. Ackermann et al. (2002) exposed rainbow trout from fertilization until one year of age to environmentally relevant concentrations of nonylphenol. Although this treatment led to induction of VTG and zona radiata proteins, it did not alter gonad morphology or sex ratio, suggesting that environmentally realistic concentrations of nonylphenol may have no adverse effects on sexual development of rainbow trout. However, Ashfield et al. (1998) found that exposure of all-female rainbow trout from hatching until 22 or 35 dph, respectively, with low concentrations (1-30 μg/L) of nonylphenol and other alkylphenols significantly impaired gonad growth. This effect persisted after termination of exposure until 466 dph. Whether the gonads were sex-reversed or not has not been analyzed in that study. Indications that sex

reversal appears to occur in feral salmonid populations has been provided by Nagler et al. (2001) from a study on wild chinook salmon in the Columbia River, northwestern US. This population showed a high incidence (84%) of a genetic marker for the Y chromosome in phenotypic females (whether the fish were exposed to EDCs was not investigated in that study). Overall, despite our good knowledge on zootechnical sex reversal in farmed salmonids, we currently have no clear understanding whether environmental concentrations of EDCs are effective to induce sex reversal or to significantly impair functional development of the gonads in salmonid species.

A gonochoristic species frequently used as model species in studies on EDCs is the medaka which develops as a differentiated gonochorist, with gonad sexual differentiation taking place shortly after hatching. Female and male phenotypes can be distinguished based on the activity of their PGCs within 24 h after hatch: those PGCs which enter mitosis are identified as oogonia, while they are referred to as spermatogonia in those larvae where no division takes place (Satoh, 1974). Exposure of developing medaka to (xeno)estrogens induces phenotypic feminisation. Whether the gonads exhibit full sex reversal or develop testis-ova appears to depend on the concentration of the (xeno)estrogens as well as on timing and length of exposure. Short-term exposure (21 days) of mature medaka to concentrations between 63.9 and 488 ng EE2/L resulted in testis-ova formation but not in sex reversal (Seki et al., 2002). In long-term developmental exposures (from hatch until 2 or 3 months of age, or full life cycle exposures), 100 ng EE2/L induced complete gonadal feminisation, but concentrations of ≤ 10 ng EE2/L or exposure to weak estrogenic compounds such as nonyl- or octylphenol did result in intersex (testis-ova) formation instead of complete sex reversal (Gray and Metcalfe, 1997; Scholz and Gutzeit, 2000; Knörr and Braunbeck, 2002; Seki et al., 2003; Balch et al., 2004). Threshold concentrations for full sex reversal appear to be higher in medaka than in many other fish species. For instance, while 3 and 4 ng EE2/L are sufficient to induce 100% feminisation in zebrafish and fathead minnow, respectively (Länge et al., 2001; Fenske et al., 2005), it took 100 ng EE2/L to achieve full sex reversal in medaka (Scholz and Gutzeit, 2000).

The importance of the timing of EDC exposure has been shown by Gray et al. (1999) who exposed male medaka to 100 μg octylphenol/L, with the treatments starting at 1, 3, 7, 21 or 35 dph, respectively. The

incidence of testis-ova in 100-day-old fish was highest in the 3 dph group and declined when exposures were initiated with older fry. This observation can be related to the critical period of sexual differentiation which takes place around or shortly after hatching of medaka (Satoh, 1974). Further studies confirm that medaka is highly sensitive to estrogen during this developmental period: Hartley et al. (1998) exposed medaka shortly after hatch for only 48 h to 4 or 29.4 μg E2/L which led to higher percentages of females after a 2-week-grow out period. A more sophisticated time scheme has been tested by Koger et al. (2000) who treated medaka embryos of stage 10 as well as 1-, 7- and 21-day-old larvae for 6 days with 15 μg E2/L. After 5-month rearing in control water, the sex ratios were significantly biased towards females when estrogen exposure had taken place during the stage 10 or the 1- or 7-day post-hatch period, while exposure of 21-day-old medaka induced no alteration of sexual differentiation. Testis-ova were observed in all treatments, including the 21-day post-hatch group. Nimrod and Benson (1998) exposed medaka to 0.01 – 1.66 μg E2/L from hatch until the first month of age and found that all concentrations led to exclusively female populations (while nonylphenol at concentrations upto 1.9 μg/L or methoxychlor at concentrations up to 2.3 μg/L were not able to induce changes of sex ratio). Papoulias et al. (1999) administered EE2 by microinjection into the egg. The injected EE2 was present for 8 days before and 4 days after hatch, i.e. during the critical period. Microinjection of 0.5 – 2.5 ng EE2/egg caused phenotypic sex reversal of genetic males into females, with 2.5 ng/egg being the most effective dosis. Interestingly, testis-ova were not found.

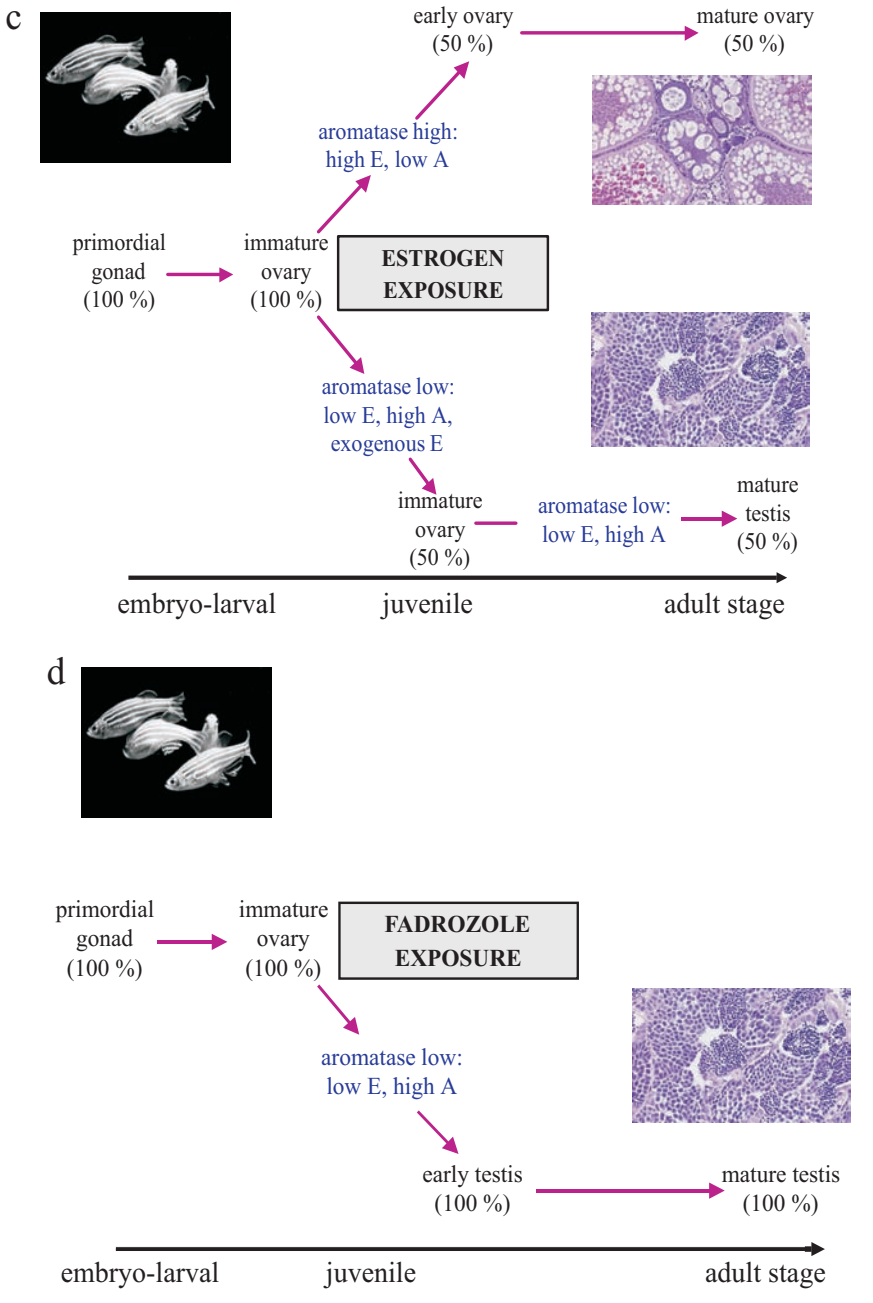
The influence of complex endocrine-active samples from the environment on sexual differentiation of a gonochoristic fish species has been studied by Rodgers-Gray et al. (2001). This work was motivated by the observation of a high incidence of intersex gonads in wild roach, *Rutilus rutilus*, from British rivers receiving estrogen-active effluents from sewage treatment plants. The authors examined whether developmental exposure to an estrogenic effluent is able to alter morphological gonad development. Juvenile roach were reared from 50 to 150-200 dph in 0, 12.5, 25, 50 or 100% estrogen-active effluent. At 50 dph, gonads of control fish were sexually undifferentiated and consisted of PGCs and somatic tissue. In control fish of 100 or 150 dph of age, the early gonad was connected to the mesentery either by a single point of attachment—indicative of a male—or by two points of attachment indicative of an

ovarian cavity and thus a female. Fish exposed to effluent dilutions showed a dose- and time-dependent increase of fish with female-like reproductive ducts, with the 100% effluent treatment leading to 100% female-like ducts. Male germ cell differentiation remained incomplete within the exposure period studied. While in the control groups, 26.7% of the fish displayed primary oocytes at 150 dph, in the exposed groups a significantly higher percentage of fish with oocytes was observed (55.2% in the group exposed to 100% effluent). Continued exposure to 100% effluent until 200 dph did not change the situation. The results indicate that exposure to the estrogenic effluent leads to a dose- and time dependent feminisation in roach. Remarkably, none of the experimental fish developed intersex gonads, as had been observed in feral roach collected from field sites impacted by estrogenic effluents. The reason for this difference is not clear as yet. Possibly, the exposure period was not long enough (i.e. mismatch with the critical period), since full morphological differentiation of male and female gonads in roach occurred only beyond 200 dph, and the induction of oocytes in testicular tissue may require exposure during the period of testicular differentiation.

To date, most studies have focused on the impact of estrogens on gonadal sex development of fish developing as differentiated gonochorist. Less emphasis has been given to the study of species with different developmental patterns. Some experimental data are available for the zebrafish, an undifferentiated gonochorist. As described above, this species initially develops an—immature, non-functional—ovary in all individuals, and only later, about 50% of the fish show degeneration of the immature ovaries and development of testes (Fig. 24.3a). Life cycle exposure of zebrafish to 3 ng EE2/L resulted in 100% feminization (Fenske et al., 2005). This effect level agrees well with EE2 threshold levels reported for full feminization of a differentiated gonochorist such as the fathead minnow (Länge et al., 2001). Interestingly, however, the feminized zebrafish population contained both individuals with mature ovaries (showing all oocyte maturation stages) and individuals with immature ovaries (showing only pre-vitellogenic oocytes) (Fig. 24.3b). How to understand this bimodal response ? A hypothetical explanation comes from the development of zebrafish as undifferentiated gonochorist. The decision whether an individual at the juvenile all-female stage continues to develop as a female or changes into a male is probably mediated through differential aromatase activity, i.e., prospective females show high aromatase activity and the resulting high estrogen levels trigger ovarian

Fig. 24.3 Contd.

Fig. 24.3 Contd.



developments, while the absence of high estrogen levels due to low aromatase activity in the prospective males promotes testicular development. If now a male fish during the transition period is exposed to exogenous estrogens, this may result in elevated internal estrogen levels. As a consequence, the fish maintains the immature ovary and arrests testis development. This would explain why adult zebrafish continuously exposed to EE2 show both individuals with mature ovaries (genetic females which have developed normally) and individuals with immature ovaries (genetic males arrested at the juvenile hermaphroditic stage).

From a life cycle experiment it is not possible to decide if effects of EDCs on gonadal differentiation are set during the critical period and became manifest only at the mature stage, or if they were caused by the continuous exposure. Therefore, Maack and Segner (2004) treated zebrafish during specific life stages, namely during: (i) the 'all-ovarian stage'; (ii) the transition stage from the ovarian gonad to the early testis; or (iii) the premature stage. Exposure to 10 ng EE2/L during either the hermaphroditic or the premature stage had no significant effect on gonad morphology or reproductive capabilities at the mature stage, while exposure during the transition period led to reduced fertilization success of adult fish, although neither gonad morphology nor sex ratio were

Fig. 24.3 Hypothetical role of aromatase in gonad differentiation of zebrafish, an undifferentiated gonochorist (juvenile hermaphrodite). **(a)** Controls. Juvenile zebrafish go through a stage where all individuals display immature, non-functional ovaries (juvenile hermaphroditic stage). Only afterwards, transition of the ovaries into testes takes place in about 50% of the individuals. This transition may be triggered/accompanied by declining activities of gonadal aromatase which, in turn, leads to elevated levels of endogenous androgens (A), but low levels of estrogens (E). **(b)** Continuous exposure to environmental estrogens. The exogenous supply of estrogens changes the E/A ratio in fish potentially transforming into males, and thereby blocks transition from the ovarian into the testes stage, i.e. the gonads are arrested at the juvenile hermaphroditic stage. Thus, in adults, both individuals with mature ovaries (females) and immature ovaries (arrested males) are present. **(c)** Exposure to environmental estrogens only during the gonad differentiation stage. Exogenous supply of estrogens changes E/A ratio in fish potentially transforming into males, and thereby blocks transition from the ovarian into the testes stage, i.e. gonads are arrested at the juvenile hermaphroditic stage. However, when the exogenous estrogens are removed, normal testicular differentiation can be resumed. Thus, in adults, both individuals with mature ovaries and with mature testes are present. **(d)** Exposure to fadrozole during gonad differentiation stage. Endogenous synthesis of estrogens is inhibited and all individuals develop testes. This change in gonad differentiation is irreversible, since when fadrozole is removed, reversal into ovaries does not occur, but all individuals develop mature testes. Results on morphological gonad differentiation based on data of Fenske and Segner (2004), Maack and Segner (2004) and Fenske et al. (2005).

altered compared to controls. Notably, when gonad morphology was examined at the termination of developmental exposure (70 dph, at the end of the transition stage), 100% of the exposed fish diplayed ovaries, while at the adult stage (> 100 dph), an approximately 1:1 ratio of fish with ovaries and with testis was observed (Fig. 24.3c). Apparently, a 100% feminization had taken place during exposure and this 'feminization' was morphologically reversed during subsequent rearing in clean water. The morphological recovery was not accompanied by a functional recovery, as indicated from a reduced fertilization success of the developmentally exposed zebrafish. Congruent observations have been reported by Hill and Janz, 2003: when they exposed zebrafish to 10 ng EE2/L from hatching until 60 dph, i.e., over a period which includes gonad differentiation, they found 100% feminization at exposure termination, while fish reared subsequently (from 60-120 dph) in clean water, showed no significant departure from the 1:1 female:male ratio. These findings suggest that exposure of zebrafish to estrogenic chemicals during gonad sexual differentiation does not irreversibly alter phenotypic gonadal sex—an observation that is in contrast to the findings on salmonids or medaka. Also intersex, a morphological response observed in estrogen-exposed medaka, salmonids or roach (see above), does not appear to be estrogen-dependent in zebrafish (Hill and Janz, 2003; Örn et al., 2003: Segner et al., 2003; Weber et al., 2003; Maack and Segner, 2004; Fenske et al., 2005). An interesting "exception" to this statement appears to be the study of Andersen et al. (2003) who exposed zebrafish to 15.4 ng EE2/L over different time periods from hatch until 60 dph and examined gonad histology at termination of exposure. Full feminization was obtained in the groups treated with EE2 from 0-60 dph or from 20-60 dph, while fish exposed for shorter periods showed variable incidence of intersex. The morphological picture of the intersex gonads, however, was different from typical testis-ova, and resembled gonads in the transition stage from the hermaphroditic ovary to the early testis. Therefore, the authors hypothesized that these gonads do not represent feminized testis but transition gonads, with the estrogen treatment apparently leading to a delay of the transition process.

While treatment of zebrafish during the period of gonad differentiation with estrogen is not able to induce irreversible phenotypic feminisation (Hill and Janz, 2003; Maack and Segner, 2004), treatment during that period with the aromatase inhibitor fadrozole leads to irreversible masculinization of the gonads (Fenske and Segner, 2004) (Fig.

24.3d). Possibly, the inhibition of aromatase activity and the resulting decline of endogenous estrogen promotes differentiation of the gonads of all individuals into testes. Since 'low estrogen' is probably the organizing signal for testicular development (see Nakamura et al., 2003), this effect may result in irreversible male differentiation of the gonad, contrary to the arrestment of male differentiation, as evoked by estrogen exposure (see above).

Effect of androgen receptor-binding substances on gonad differentiation of teleost fish

Endocrine disruption may occur through interference of environmental substances with androgen-signaling pathways and ligand binding to the androgen receptor (AR). As with estrogens, steroideal ligands for this receptor with high potency and *in vivo* stability have been developed as pharmaceutical agents (e.g., phtalimide). Further, a number of non-steroideal compounds are able to bind to the AR, including pharmaceuticals for treatment of prostate cancer such as flutamide, or pesticides such as vinclozolin (Katzenellenbogen and Muthyala, 2003). For certain compounds such as the weak estrogenic o,p´-DDE, it is discussed that its endocrine disruptive activity is not due to its estrogenic but to an anti-androgenic effect (Katzenellenbogen and Muthyala, 2003). Wastewater from wood pulping has been shown to cause masculinization in fish, with the causative agents probably being wood-derived AR ligands (Bortone and Cody, 1999).

Developmental differentiation of fish gonads can be altered by androgen-active substances. For example, exposure of medaka or zebrafish during the critical period of development to the synthetic androgen methyltestosterone results in 100% male populations (Papoulias et al., 2000; Örn et al., 2003). Aromatizable androgens such as methyltestosterone can induce 'paradoxical feminisation' (Fenske and Segner, 2004), but this is a concentration-dependent phenomenon, as shown by Örn et al. (2000; 2003): while methyltestosterone concentrations up to 1 μg/L led to masculinization of developing zebrafish, concentrations of 10 μg/L and higher resulted in feminization.

The effect of antiandrogens on sexual differentiation has been investigated by Kiparissis et al. (2003): medaka were exposed to the androgen receptor antagonists, cyproterone acetate and vinclozolin, during a three-month period which included the critical window of sexual

differentiation. The anti-androgenic substances did not induce sex reversal but gave rise to a low incidence of testis-ova and a moderate inhibition of oogenesis and spermatogenesis.

Effects of substances interfering with steroid metabolism on gonad differentiation of teleost fish

Alternative to receptor binding, environmental substances can disrupt endocrine processes through interference with endogenous hormone metabolism, i.e., via modification of the hormone kinetics (e.g., by competing for steroid-binding globulin; Kloas et al., 2000; McLachlan, 2001) or by stimulation or inhibition of enzymes responsible for the synthesis and degradation of the hormones. One class of environmental contaminants, which is of particular interest in this respect, are inhibitors of CYP19 aromatase. This enzyme converts androgens into estrogens. Aromatase inhibitors include pharmaceuticals, e.g., fadrozole, which is used in therapeutic treatment of breast cancer, pesticides, e.g., tributyltin (Shimasaki et al., 2003), or imidazole compounds (Monod et al., 2004). Also, wood-derived substances in pulp mill effluents are obviously able to disturb steroid synthesis (McMaster et al., 1995).

The potential of disruptors of steroidogenesis to alter gonad differentiation of fish has been demonstrated for zebrafish, using fadrozole as a model substance (Fenske and Segner, 2004, see above). Another example is provided by tributyltin, a substance used in antifouling paints for ships and, therefore, widely distributed in the aquatic environment. This compound is considered to inhibit aromatase activity and to cause imposex in mollusks (Oehlmann and Schulte-Oehlmann, 2003). Shimasaki et al. (2003) examined the effect of tributyltin on the sex-differentiation process in genetically female Japanese flounder, *Paralichthys olivaceus*. The fish were fed an artificial diet containing tributyltin at concentrations of 0.1 and 1.0 μg/g diet in the period from 35-100 dph, which includes the period of sex differentiation. This treatment led to a significant increase of sex-reversed males showing histologically normal testes. McAllister and Kime (2003) tested environmental concentrations of waterborne tributyltin (0.01-100 ng/L) in the zebrafish. The fish were exposed to tributyltin from 0-70, 0-30 or 30-60 days post-fertilization (dpf), with the first two periods including the period of sexual differentiation. In the 0-70 dpf exposures, the sex ratio became significantly male-biased at concentrations of 0.1 ng/L or higher, while in

the 0-30 dpf exposures, it took concentrations of 1 ng/L and higher to significantly change the percentage of males. When tributyltin exposure was restricted to the period from 30 and 60 dpf, a significant shift to males occurred only at the highest concentration tested (100 ng/L). These results confirm that zebrafish is particularly susceptible to steroidogenesis-interfering substances during the critical period of gonad differentiation.

INFLUENCE OF ENDOCRINE DISRUPTORS ON THE FISH IGF SYSTEM

There is preliminary evidence that estrogens such as E2 may interfere with the essential growth hormone (GH)/insulin-like growth factor I (IGF-I) system of fish. IGF-I is mainly produced in fish liver—the principal source of the circulating (endocrine) IGF-I—under the influence of GH. IGF-I released from the liver into the circulation exerts growth-promoting effects on a variety of target cells. In addition, IGF-I is also expressed in numerous other organs and most likely stimulates organ-specific functions of parenchymal cells by paracrine/autocrine mechanisms. There is increasing evidence that GH stimulates the expression of IGF-I also in fish extrahepatic sites (see: Reinecke, this volume).

Two recent studies have dealt with the potential influence of E2 on fish liver IGF-I. The *in vitro* effects of E2 on the production of IGF-I and IGF-binding proteins (IGFBPs) were examined using primary cultured hepatocytes of the female and male tilapia (Riley et al., 2004). In this study, 10 and 100 μM E2 produced a significant stimulation of VTG release and a concomitant decrease in IGF-I mRNA expression without obvious differences between hepatocytes from male and female individuals. E2 also affected the release of the investigated IGFBPs (see: Kelley et al., this volume), i.e., a 25 kDa and a 30 kDa form. In male hepatocytes, E2 significantly reduced the release of the 25 kDa binding protein but stimulated the release of the 30 kDa IGFBP. In contrast, E2 significantly increased both the 25 and the 30 kDa IGFBP in female hepatocytes. Therefore, in this study on tilapia hepatocytes, E2 affected both the hormone IGF-I and IGFBPs, although in different ways.

Evidence for an interaction of E2 with the synthesis of liver IGF-I has also been obtained by an experiment using the common approach to induce feminization in fish by treatment with E2 during the sensitive period of gonadal sex differentiation. A balanced population of tilapia was fed during 10-40 dpf with E2 at the optimal dosage (125 μg E2/g food) so

as to induce functional feminization in most individuals, and the effects were studied 1.5 months after completion of the treatment (Shved et al., 2004). At the end of the experiment, the treated group consisted of a majority of females (83%). A significantly lowered expression of IGF-I mRNA was observed in liver of E2-treated females by about 45%, and in males by about 60%. Thus, in contrast to the above hepatocyte culture study (Riley et al., 2004), the E2 effect was gender-specific. In agreement with the reduced expression of the IGF-I gene in liver, serum IGF-I levels showed a slight decrease in the E2-treated group (14.4±2.3 ng/mL) when compared to the untreated siblings (16.7±3.3 ng/mL). This preliminary study indicates that E2 seems to exert long-term effects on the endocrine IGF-I system.

There is also some indication that in addition to the natural estrogen, E2, also xenoestrogens may interact with IGF-I in fish cartilage and bone growth. The *in vitro* effects of estrogenic compounds such as EE2, diethylstilbestrol, genistein, and nonylphenol, were studied on tilapia cartilage (Ng et al., 2001). Cartilage explants were cultured with the estrogens with or without mammalian IGF-I. At higher (10 μg/mL) than physiological (3-6 ng/mL) levels, the xenoestrogenic compounds were able to inhibit sulphate and thymidine incorporation. The inhibiting actions were obtained both on the low basal level of incorporation and on the higher uptake induced by IGF-I. Fish exposed to environmental estrogens often suffer from growth retardation, even at low exposure concentrations. On the one hand, this effect may reflect a general toxic stress situation where growth slows down due to the enhanced energetic needs for toxicant acclimation. On the other hand, the growth-retarding effect may also reflect an interference of estrogens with circulating IGF-I and/or IGF-I expressed in cartilage. A direct effect of bisphenol A on osteoclasts and osteoblasts has been shown using a culture system of goldfish scales (Suzuki and Hattori, 2003), using tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as markers of osteoclasts and osteoblasts, respectively. Bisphenol A at 10^{-5} M significantly suppressed both TRAP and ALP activities *in vitro* and *in vivo* and decreased the expression of IGF-I mRNA in cultured scales. However, E2 stimulated both TRAP and ALP activities and did not alter IGF-I mRNA expression, suggesting that bisphenol A and E2 may differently affect bone metabolism (Suzuki and Hattori, 2003). Furthermore, the xenoestrogen

bisphenol A more pronouncedly interacted with local IGF-I in bone than did E2.

A further physiological process in fish where estrogens and xenoestrogens may interfere with the GH/IGF-I system is smoltification, i.e., the developmental stage through which the stream-dwelling parr is transformed into the seaward migrating smolt. Here GH plays a central role likely both by direct and indirect action, the latter being mediated via endocrine or local IGF-I on the expression of gill Na^+, K^+-ATPase (see: Reinecke, this volume). In agreement with the endocrine function of IGF-I, serum IGF-I levels are considered to be reliable indicators of smolt quality. Recent evidence indicates that the role of IGF-I in smoltification can be modulated by estrogens. E2 (100 ng/L) and 4-nonylphenol (20 µg/L) significantly reduced plasma IGF-I by about 25% in Atlantic salmon during smoltification (Arsenault et al., 2004), and it has been assumed that environmental xenoestrogens such as 4-nonylphenol have contributed to the historical decline of anadromous salmons in eastern Canada (Fairchild et al., 1999).

Some evidence indicates that E2 and xenoestrogens may also interact with IGF-I in fish gonads. Loomis and Thomas (1999) showed that the ER of Atlantic croaker testes has a 5- to 10-fold higher affinity for xenoestrogens than the hepatic ER. An effect of different quality was reported by Le Gac et al. (2001) who observed that the estrogenic nonylphenol diethoxylate displaced IGF-I from its receptor of isolated testicular cells from rainbow trout. However, since the same effect was also observed for the non-estrogenic chemical prochloraz, it may represent more a detergent than an estrogenic activity. In the above described E2-feeding experiment (Shved et al., 2004), IGF-I mRNA in female gonads was found to be decreased by about 30%, and in male gonads by about 70%, the latter likely being the cause for the impaired testes development observed.

Thus, to date, only a few studies have addressed the question of possible interferences between EDCs and the IGF-I system of fish. Provided that the preliminary data are strengthened by further studies, E2 and estrogenic compounds must be considered to have a higher impact as endocrine disruptors as yet thought because they interfere with a key hormonal system that is involved in numerous processes of metabolism, reproduction, differentiation, growth, development and aging.

INTERACTIONS BETWEEN THE ENDOCRINE AND THE IMMUNE SYSTEMS IN FISH

Intensified commercial aquaculture goes along with increasing spread of diseases in fish populations. Thus, growing interest arises in the immune system in fish. Whereas earlier the endocrine and the immune system were considered as two separated entities, today there are increasing insights into their mutual interaction. New information about the interaction between the immune system and the hypothalamic-pituitary-interrenal (HPI) axis may provide a target for pharmacological intervention (Yada and Nakanishi, 2002).

Similarities and analogues to the constituents of the mammalian immune system comprise one focus of interest in fish immunology research (Trede et al., 2004) as well as differences and peculiarities which are even more pronounced due to the diversity between the fishes themselves. Although there exist morphological, functional, and molecular analogies among mammalian and fish immunologic features which are currently being investigated with growing interest, various differences are apparent (see: Yada and Nakanishi, 2002; Traver et al., 2003). Recently, Trede et al. (2004) underlined the importance of a zebrafish gene library for potential insights into the mammalian innate and adaptive immune system. Because zebrafish is transparent, it might provide an elegant tool to monitor green-fluorescent protein-labelled T cells to analyze *in vivo* the potential influence of irradiation and dexamethasone treatment (Langenau et al., 2004). This chapter reviews the major components of the fish immune system, considering macroscopical, microscopical and functional aspects.

Macroscopic and Microscopic Approach to Immune Organs

Fish do not possess bone marrow tissue (Press and Evensen, 1999); instead, the head kidney, together with other organs are considered as equivalents in bony and cartilaginous fish to mammalian bone marrow (Torroba and Zapata, 2003). The head kidney represents the major lymphoid organ. The lymphoid tissue in the head kidney develops around the branches of the posterior vein and consists of a fine network of reticular cells filled with immature and mature blood cells (Endo and Oguri, 1995; Suzuki, 1995). Most recently, blood lineages from head kidney have been separated using cytofluorometry techniques (Traver et

al., 2003; Trede et al., 2004). Lymph nodes are lacking from bony and cartilaginous fish (Press and Evensen, 1999), and gut-associated lymphoid tissues are not well developed (Suzuki, 1995).

Elasmobranchs and teleosts dispose over thymus and spleen, whereas these organs are not yet developed in cyclostomes (Yada and Nakanishi, 2002). The thymus is a branchiogenic organ (Suzuki, 1995) and seems to be the first lymphoid organ to arise during teleost development (Hansen and Zapata, 1998). The thymus is a paired organ at the dorsolateral wall of the pharynx, close to the branchial chamber. The stroma mainly consists of reticular cells and lymphocytes. Few studies have dealt with the thymus in cartilaginous fish. The nurse shark, *Ginglymostoma cirratum*, possesses a prototypic thymus with a cortex and medulla that contains macrophages, dendritic cells and lymphocytes expressing all T cell receptor (TCR) genes identified in mammals (Rumfelt et al., 2002). In bony fish, recent studies revealed, for carp, a clear distinction between cortex and medulla (Romano et al., 1999), and for zebrafish regions with TCRα gene expression accompanied by diminished *rag1* expression indicating preferential T cell regions (Danilova et al., 2004). In flounder, no inner and outer zones were visible even at the latest stage before involution (Liu et al., 2004). Moreover, only about 60% of the flounders investigated exhibited a thymus at the age of 6 months, i.e., when, thymus involution starts.

Although fish spleen shows lymphocyte-rich (white) and erythrocyte-rich (red) portions, the boundary is not as clear as in mammals (Suzuki, 1995), and in flounder spleen, Liu et al. (2004) did not identify separate red and white pulp.

Cellular and Humoral Immune Systems

Despite the aforementioned differences to mammalian lymphoid tissues, bony and cartilaginous fish are able to perform specific immune responses comparable to mammals. Thus, immunity against pathogens, including bacteria, parasites, and viruses in fish is mediated via cellular and humoral immune responses (Somamoto et al., 2002).

Fish possess a variety of immune cells including monocytes, macrophages, granulocytes and lymphocyte populations similar to T and B cells (Ellis, 1999). Whereas teleosts and elasmobranchs are known to dispose over T and B lymphocytes, macrophages and dendritic cells

(Rumfelt et al., 2002), the existence of lymphocytes and plasma cells is doubtful in cyclostomes (Yada and Nakanishi, 2002).

Teleosts and elasmobranchs possess antigen presentation and recognition molecules, such as major histocompatibility complex (MHC) and TCR (Nakanishi et al., 2002). Recently, diverse molecules involved in specific antigen recognition and response such as MHC, TCR, and cytokines could be characterized for some teleostean and elasmobranchian species (Daniels et al., 1999; Nakanishi and Okamoto, 1999; Partula, 1999; Secombes et al., 1999; Saeij et al., 2003). However, further characterizations of T cell subsets are missing due to the lack of reliable detection techniques for fish surface markers, such as TCR and clusters of differentiation (CD) (Yada and Nakanishi, 2002), but modern approaches will allow the investigation of fish lymphatic cells (reviewed by Yoder, 2004). Recently, peripheral blood leucocytes (PBL) could be characterized as CD8+ lymphocytes by magnetic sorting and subsequent RT-PCR (Fischer et al., 2003) and the MHC I protein was visualized by immunohistochemistry in rainbow trout (Fischer et al., 2005).

Teleost fish are capable of a variety of immunologic reactions such as antibody production, graft versus host and delayed hypersensitivity reactions, as well as mixed leucocyte reactions (Nakanishi et al., 1999, 2002). Like in mammals, immunity against bacteria is also provided by unspecific mechanisms such as lysins, complement factors with lytic, proinflammatory, chemotactic and opsonizing functions, primarily executed by neutrophilic granulocytes and macrophages. Additionally, fish dispose over a specific humoral defence system where antibodies act as opsonins to enhance cellular response. Although some fish-pathogenic bacteria like *A. salmonicida* and *R. salmoninarum* have a variety of protection systems, concerted efforts of the complex immunologic system in teleost succeed in killing these bacteria (Ellis, 1999). Even in zebrafish embryos, the functional capacity of macrophages to encounter bacterial infection was detectable (Herbomel et al., 1999).

Very recently, phylogenetic trees have been created for tumor necrosis factor (TNF)-α (Savan and Sakai, 2004) and the interleukin (IL)-1 family (Huising et al., 2004). The cytokine system is considered as an ancient paracrine system that exhibits low homologies (e.g., 30% for IL-1β and TNF-α) among vertebrate species on the amino acid level. However, the secondary and tertiary structures are conserved to a higher degree (Secombes et al., 1998). The low conservation degree of cytokines at the

amino acid level is opposite to the phylogenetic conservation of hormones (Engelsma et al., 2002).

In summary, cartilaginous and bony fish possess numerous elements of the immune system similarly to mammals. A differentiated adaptive immune system comparable to that in mammals seems to have arisen with the upcome of the lowest vertebrates (Yada and Nakanishi, 2002).

Interaction Between the Neuroendocrine and the Immune Systems

Cortisol and Catecholamines

Elevated cortisol levels after stress are a typical response of fish, as observed, for instace, after handling stress in *S. salar* (Leloup-Hatey, 1964), in subordinate individuals of rainbow trout (Larson et al., 2004) and arctic charr (Elofsson et al., 2000), but also after bacterial infection in *O. nerka* (Williams et al., 1977) and in moribund vibriosis-infected seabream (Deane et al., 2001). Generally, the regulation axis for corticoid production is similar in fish and in mammals (Fig. 24.4, see Wendelaar Bonga, 1997). However, the regulation of the axis differs in some essential aspects between fish and mammals (see Weyts et al., 1999; Harris and Bird, 2000). Due to the lack of adrenal gland in fish, the major source of corticosteroids and catecholamines in teleost fish is the head kidney, i.e. the anterior part of the kidney (Endo and Oguri, 1995). Herein, the interrenal cells produce corticosteroids and, thus, are comparable to the mammalian adrenal cortex, while the chromaffin cells secrete catcholamines (Wendelaar Bonga, 1997; Weyts et al., 1999).

While the involvement of peripheral and central catecholamines in neuroendocrine-immune interactions has been the focus of interest in mammals for several decades (Livnat et al., 1985), only a few studies in fish exist. In carp, the sympathetic system has been found to act on the chromaffin cells and the parasympathetic system on the interrenal cells (Gfell et al., 1997). Indications for paracrine effects were also described (Wolfensberger et al., 1995; Gfell et al., 1997). In cyclostomes, the catecholaminergic cells are dispersed throughout the peritoneal cavity in association with large blood vessels and concentrated in the walls of the systemic and portal hearts (Bloom et al., 1962; Epple et al., 1985), but in chondrichthyes, the chromaffin cells are found in association with paravertebral autonomic ganglia (Shepard et al., 1953). In teleost fish, the

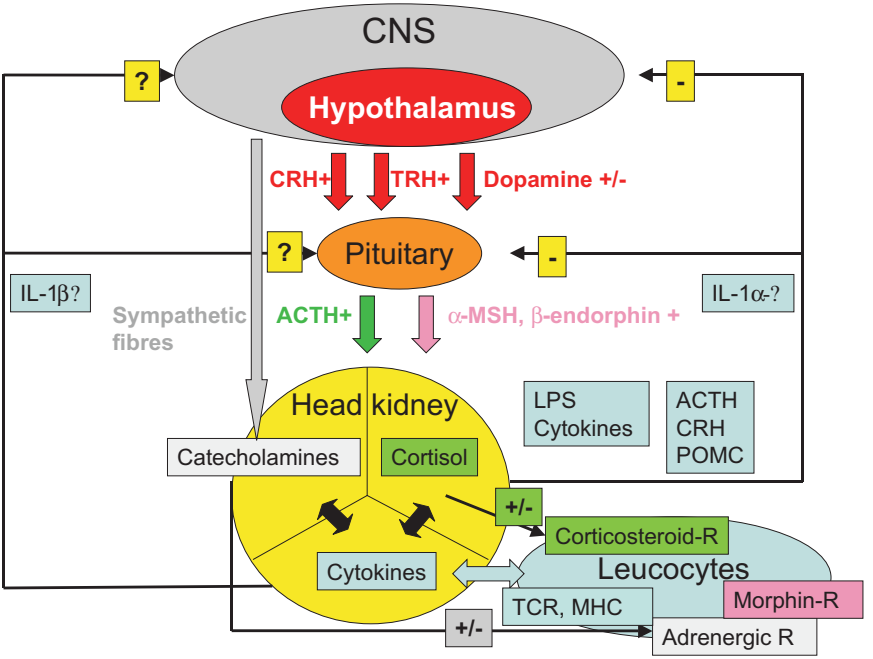


Fig. 24.4 Potential interaction of relevant elements of the neuro-/endocrine system with the immune system in teleosts. Modified from Cooper and Faisal (1990), Weyts et al. (1998c; 1999), Harris and Bird (2000), Watanuki et al. (2000; 2003b), Engelsma et al. (2002), and Yada and Nakanishi (2002). ACTH: adrenocorticotropic hormone. CNS: Central nervous system. CRH: Corticotropin-releasing hormone. IL: Interleukin. LPS: Lipopolysaccharide. MHC: Major histocompatibility complex. MSH: Melanocyte-stimulating hormone. POMC: Proopiomelanocortin. R: Receptors. TCR: T cell receptor. TRH: Thyroxin-releasing hormone.

chromaffin cells which synthesise catecholamines (Fig. 24.5) are intermingled with the steroidogenic interrenal cells in close association to the lymphoid tissue of the head kidney (Wolfensberger et al., 1995; Reid et al., 1998). They, thus, constitute the fish analogue to the adrenal medulla in higher vertebrates.

Both cortisol and catecholamines are considered as main stress mediators in fish. Consequently, the head kidney plays a key role in the complex stress response system in fish. This importance finds its expression in the central role of the HPI axis in fish (Fig. 24.4). Generally, the effects of cortisol on the immune system in fish—whether occurring exogenously through cortisol application, or endogenously due to stress activators—are comparable to those in mammals. In brief, cortisol effects

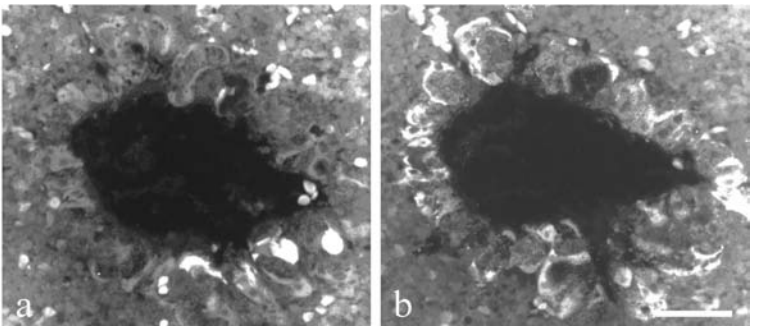


Fig. 24.5 Carp head kidney blood vessel wall containing cells immunoreactive for **(a)** dopamine-ß-hydroxylase and **(b)** PNMT indicating catecholamine synthesis. Bar: 60 µm.

comprise decreased numbers of circulating or intraperitoneal leukocytes, impaired proliferation of lymphocytes and antibody production (Fletcher, 1986; Weyts et al., 1999; Milston et al., 2003b), and apoptosis of B cells (Weyts et al., 1998a; Verburg-van-Kemenade et al., 1999, Harris and Bird, 2000). In contrary, no change or increase of neutrophilic granulocytes was observed (Ainsworth et al., 1991), probably due to cortisol-supported rescue from apoptosis (Weyts et al., 1998b). The glucocorticoid receptor was characterized in PBL of carp (Weyts et al., 1998c), in the upper epidermal layer of pavement and filament cells of rainbow trout, and, furthermore, in leukocytes migrating into these layers, thus, directly at the site of parasite attachment (Haond et al., 2003). The latter authors observed a reduced number of PBL in cortisol-pretreated rainbow trout. Glucocorticoid-induced immuno-suppression followed by higher susceptibility to infectious agents has been observed for many species and pathogens (Harris et al., 2000).

Cortisol application depressed leukocyte phagocytic activity *in vitro* in a dose-dependent manner, whereas no effect was observed after catecholamine treatment. Combined treatment with cortisol and a catecholamine-antagonist even enhanced the suppressing effects on phagocytosis (Chen et al., 2002). The physiology of the adrenergic stress response in fish has been reviewed by Fabbri et al. (1998). Cortisol does not seem to elevate phenylethanolamine-N-methyltransferase (PNMT, catalyses conversion of noradrenalin to adrenalin) *in vitro* in rainbow trout, suggesting other pathways for the influence of cortisol on catecholamines (Jönsson et al., 1983) which are still unknown. This contrasts the situation in mammals, where cortisol elevates the levels of

adrenaline in the adrenal gland (Wurtman et al., 1967) by enhancement of PNMT activity. Nevertheless, an increase of adrenalin was observed after handling stress in Pacific salmon (Demers and Bayne, 1997) and increased levels of catecholamines were also observed after confrontation of subdominant fish with dominant fish as social stress factor (Cooper and Faisal, 1990; Larson et al., 2004).

Cytokines

Cytokines may be also involved in the orchestration of stress mediation, infection and the HPI axis in fish. Currently, IL-1ß, known to be induced by bacterial lipopolysaccharide (LPS) and bacterial infection *in vivo*, is the most studied cytokine in fish (Zou et al., 2000). IL-1β significantly elevated plasma cortisol in a dose- and time-dependent manner, and the effect could be blocked by suppression of ACTH, which indicates an influence of IL-1β on the HPI axis (Holland et al., 2002). In contrary, using semiquantitative RT-PCR in a trout model infected with *Tetracapsuloides bryosalmonae*—causative agent of proliferative kidney disease—Holland et al. (2003) found upregulations of COX-2, TNF-α2, and TNF-β1, but not of IL-1β-1 and -2, IL-8 and TNF-α1. Stress and infection result in production of cytokines which may influence the endocrine function and vice versa.

The interaction of HPI axis hormones and immune cells

Fish leukocytes produce hormones of the HPI axis. Corticotropin-releasing hormone (CRH) and proopiomelanocortin (POMC) mRNAs were detected in goldfish thymic cells and phagocytes (Ottaviani et al., 1995, 1998). In addition, channel catfish T and B cells were found to secrete ACTH (Arnold and Rice, 2000). Furthermore, GH and prolactin mRNA expression were found for coho salmon and rainbow trout in several lymphoid tissues such as head kidney, spleen, thymus, intestine, and PBL (Mori and Devlin, 1999; Yang et al., 1999; Yada and Azuma, 2002; Yada et al., 2002).

Vice versa, there is indication of an influence of pituitary hormones on the immune system, e.g., the prolactin receptor gene has been detected on tilapia phagocytes and PBL so far (Sandra et al., 2000; Yada et al., 2002). Prolactin and GH have been shown to enhance the mitotic and

phagocytic activity of immune cells which resulted in strengthened killing mechanisms towards pathogens (Harris and Bird, 2000; Yada et al., 2002). Elevated GH secretion during seawater adaption in rainbow trout was found to be accompanied by higher plasma lysozyme levels and phagocytic activity (Yada et al., 2001). Furthermore, decreased IgM levels in hypophysectomized rainbow trout were restored by GH (Yada et al., 1999), and GH was found to stimulate also non-specific immune parameters (Yada et al., 2001). Also superoxide anion was depressed after hypophysectomy (Yada and Azuma, 2002). For prolactin and the prolactin receptor, contradictory reports exist with respect to the effect of seawater adaptation (Auperin et al., 1995; Sandra et al., 2000), but, finally enhanced the response of O_2^- production to prolactin pretreatment.

Interaction of Endocrine Active Compounds with the Neuroendocrine-immune Axis

Influence of sex steroidal hormones on the immune system in mammals

Recently, the potential impact of sex steroids on the immune system has influenced the focus of research (Besedowsky and Del Rey, 1996). In the human immune system, a variety of modifications seem to be induced by sex steroids (Ansar Ahmed, 2000). Women are generally thought to have stronger immune capabilities than men, probably due to E2 which has been shown to have marked influence on the immune system. Also, the enhanced susceptibility of women to autoimmune disease appears to be related to gender-specific endocrine differences. E2 seems to act on all relevant compounds of the immune system since lymphoid and non-lymphoid cells possess ERs. Estrogens act directly on the immune system, e.g., bone marrow or thymus, which play central roles in the elimination of autoreactive immune cells. Further, estrogens seem to interfere with the hypothalamic-pituitary axis and the orchestration of cytokines. Also in immunoregulation, estrogens play a role by altering apoptosis behaviour of cells. It was found that estrogen-treated mice were quite resistant to cell death in vitro (Ansar Ahmed, 2000). In pregnancy, the immunosuppressive effects of progesterone are essential for survival of the fetus and protection against maternal immune cells (reviewed by Fournier et al., 2000).

Differential influence of sex steroid hormones on fish immune system

Both from field and laboratory studies, evidence is available that estrogenic or androgenic EDCs are able to disturb the immune system of fish. Exposure of roach to bleached kraft mill effluents under laboratory conditions for 21 days led to affection of immunologic parameters, with the effects in males being mainly inhibitory whereas in females both decreasing and increasing effects were evident (Aaltonen et al., 2000). Patiño et al. (2000) detected the ER-α gene in channel catfish lymphocytes, and ARs were found in salmonid lymphocytes (Slater et al., 1995), suggesting a functional involvement of sex steroids in the immune system. Slater and Schreck (1993) observed that testosterone (but not E2) altered the antibody-producing cells in chinook salmon *in vivo* and, further (1997), showed killing of leukocytes by physiological testosterone levels. In contrary, elevated testosterone levels were observed with developing vibriosis in seabream accompanied by significantly increased phagocytosis (Deane et al., 2001). Further, 11-ketotestosterone was found to enhance—but E2 to inhibit—the intracellular accumulation of proIL-1β in LPS/DNA-activated head-kidney acidophilic granulocytes in gilthead seabream (Chaves-Pozo et al., 2003).

Influence of EDCs on the immune system in fish

In order to investigate the potential effects of estrogenic compounds in fish, Wang and Belosevic (1995) examined the effects of cortisol and E2 on the inflammatory function of macrophages *in vitro* using a goldfish macrophage-derived cell line. Here, cortisol and E2 at identical threshold values suppressed chemotaxis and phagocytosis, but only cortisol also suppressed nitric oxide (NO). The authors concluded that the immunosuppressive effects of E2 may influence fish defence against infectious diseases. Similar effects were observed by Gushiken et al. (2002) who exposed carp phagocytic cells *in vitro* to low concentrations of bisphenol A and nonylphenol (0.1-10 nM) and found changes in oxidative burst and phagocytic activity whereas NO remained unchanged. In an *in vivo* study, Wang and Belosevic (1994) applied E2 by tube implantation into goldfish which led to significantly higher susceptibility to *Trypanosoma danilewskyi* and to higher mortality. E2-implanted individuals displayed impaired mitogenic activity of leukocytes which indicates a disturbed immune capacity.

Yamaguchi et al. (2001) exposed carp head kidney leukocytes *in vitro* to 1-1000 nM E2, progesterone, and 11-ketotestosterone. At a concentration as low as 1 nM, all steroids suppressed phagocytic activity. NO was suppressed by 11-ketotestosterone und progesterone, whereas the oxidative burst was not affected by any of the steroids. Cortisol expectedly suppressed phagocytic activity, NO and chemiluminescence.

Watanuki et al. (2002) investigated the potential modulation of immune parameters on phagocyte function by injecting carps with steroids. Treatment with E2, 11-ketotestosterone, progesterone, and cortisol (1 and 5 μg/kg fish), respectively, suppressed, at day 1 and 3 p.i., phagocytosis and NO in a dose-dependent manner. The strength of the sex hormone effects was comparable to that evoked by cortisol. Watanuki et al. (2003a) further investigated whether estrogenic phtalates modulate immune parameters for phagocyte function. Leukocytes from carp head kidney exposed in vitro to 1-1000 nM of phtalates displayed stimulation of oxidative burst and phagocytic activity from 100 nM onward but NO remained unchanged. The latter effect is in contrast to the earlier findings with cortisol and E2 (Watanuki et al., 2002). The authors concluded that xenoestrogens affect not only the reproductive function but also act as immune modulators. Although estrogen-like *in vitro* effects have been obtained for phtalates, it remains to be clarified if the immunomodulatory function is also relevant *in vivo* and if it goes along with increased susceptibility to pathogens.

Potential importance of species differences in fish immune systems

The finding that E2 and xenoestrogens modulate fish macrophage function suggests the potency of EDCs to act not only via the reproductive axis, but to affect wildlife populations also through modulation of their immune competence and, consequently, their resistance against infections (Jobling and Tyler, 2003b). Species differences were found by Law et al. (2001) when they investigated *in vitro* the potential roles of cortisol and gonadal steroids on phagocytic activity of PBL in tilapia and carp. Glucocorticoids suppressed the phagocytic activity of leukocytes in a dose- and time-dependent manner, in tilapia with a stronger effect than in carp. E2 suppressed the phagocytic activity at higher concentrations in tilapia but not in carp, which the authors attributed to species differences, e.g., on receptors. Testosterone and progesterone remained without effects

from which the authors explained the gender differences in disease resistance.

Also, between the cyprinid carp and the salmonid rainbow trout species, diversities were detected: Hou et al. (1999) detected immunosuppression in juvenile rainbow trout in association with lowered IgM levels after administration of cortisol, testosterone, 11-ketotestosterone, and E2. In contrary, in common carp Saha et al. (2002) found no significant effect on serum IgM when testosterone and 11-ketotestosterone in males and E2 in females increased in parallel to gonadal maturation. However, IgM levels and IgM-secreting cell numbers were high during spawning season. IgM was, thus, correlated to sexual maturation. Interestingly, elevated cortisol levels during spawning season had no suppressive effect on IgM nor on IgM-secreting cell numbers. In contrary to these findings, Suzuki et al. (1997) measured decreased IgM plasma levels in three strains of rainbow trout during spawning season in a reverse relation to steroid reproduction hormones. The detected fungal diseases in mature fish could not be explained by IgM level differences between infected and uninfected animals. However, the IgM changes became reversed by increased testosterone, and E2.

Saha et al. (2003) explored the effects of steroid hormones on immune cell apoptosis: carp leukocytes and thymic cells were cultured in vitro in the presence of cortisol, E2, testosterone, and 11-ketotestosterone, respectively, for 16 h. Then cytofluorometry was performed in order to determine the amount of apoptotic cells. Cortisol induced apoptosis, whereas gonadal steroids did not. Thus, steroid action on immune functions seems to occur through different pathways. Also IgM production was not altered in carp by gonadal steroids as found in another study (Saha et al., 2004). Here, the depressing effect of testosterone towards the IgM-secreting cells was tissue-specific while E2 and 11-ketotestosterone exerted no effects on IgM-secreting cells. Furthermore, neither E2 nor testosterone nor 11-ketotestosterone exerted any effects on IgM-secreting cells apoptosis which is in contrary to results in rainbow trout (Suzuki et al., 1997). However, in goldfish a positive relationship of plasma IgM levels with gonadal steroids was detected (Suzuki et al., 1996). The authors conclude from the detected species diversities that a lack of interaction between endocrine and immune parameters in cyprinids enables their immune system to be more effective during the spawning season than in salmonids (Saha et al., 2004). Thus, the disturbing effects

of EDCs might interfere with reproduction not only on the level of the HPG axis but, by interference with potential competition advantages, also via the HPI axis.

Immunomodulating effects of estrogenic compounds have also been reported for non-mature life stages of fish. In a study conducted on juvenile chinook salmon, Milston et al. (2003a) observed that short-term exposure to o,p'-DDE resulted in long-term suppression of IgM levels, with this effect not being sex related. Notably, however, the vehicle DMSO induced similar changes.

CONCLUSIONS

To date, the focus of endocrine disruption research has been mainly on the effects of estrogen-active substances on sexual differentiation and reproductive function. However, more consideration should be given to other endocrine-regulated processes such as growth and immune functions being potential targets of EDC action.

The assessment of EDC effects on sexual differentiation of fish has highlighted the importance to go beyond a descriptive approach in order to be able to classify and understand the various responses. Such a rational approach has to take into account the different patterns and timing of sexual development of fish (e.g., differentiated versus undifferentiated gonochorists), the different organs involved in sexual development (e.g., brain, pituitary and gonads) as well as different modes of EDC action (e.g., effects mediated through ligand binding versus effects mediated through inhibition of steroidogenesis).

Endocrine-active substances appear to interact—as suggested from several lines of evidence discussed in this review—not only with the sex steroid system of fish but also with the IGF-I system, which is a key hormonal system regulating vital processes such as differentiation, growth, development and probably reproduction (see: Reinecke, this volume). Currently, we have no clues on the mode of interaction between EDCs and the IGF-I system, nor do we know if effects of hormonally active substances on the GH/IGF (hormones, receptors, IGFBPs) system take place at environmentally realistic concentrations, and adversely affect population-relevant life history traits. Mechanisms and consequences of the EDC-IGF interaction may vary with target tissues. Since growth and sexual development are closely interrelated in fish, it may well be possible that EDCs influence sexual parameters of fish indirectly via the IGF/GH

system, or vice versa. It will be a major challenge of future research on EDCs to better address this complexity of the endocrine system and the consequences of this complexity for EDC effects.

Concerning possible EDC effects on the immune system, at present, only preliminary conclusions can be drawn. There is evidence on a close interaction between the immune system and stress mediators, cytokines and the hormones of the HPG and the HPI axis, e.g., during adaptation to salinity, temperature and spawning season. However, it is not understood at all if and how EDCs may interfere with this endocrine-immune crosstalk, although a disrupting action of environmental EDCs on the fish immune system would be of direct ecological relevance due to its compromising effect on the pathogen resistance of the fish. EDC effects in fish have attracted much attention because they affect vital functions such as development and reproduction, which are determinants of population growth and survival. Even if now growth and immune defense/disease resistance of fish are potential targets of hormonally active substances, this would have important implications for the ecological hazard assessment of EDCs.

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