Hormonal Control of Fish Euryhalinity

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HORMONAL CONTROL OF FISH EURYHALINITY

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Hormones play a critical role in maintaining body fluid balance in euryhaline fishes during changes in environmental salinity. The neuroendocrine axis senses osmotic and ionic changes, then signals and coordinates tissue-specific responses to regulate water and ion fluxes. Rapid-acting hormones, e.g. angiotensins, cope with immediate challenges by controlling drinking rate and the activity of ion transporters in the gill, gut, and kidney. Slow-acting hormones, e.g. prolactin and growth hormone/insulin-like

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growth factor-1, reorganize the body for long-term acclimation by altering the abundance of ion transporters and through cell proliferation and differentiation of ionocytes and other osmoregulatory cells. Euryhaline species exist in all groups of fish, including cyclostomes, and cartilaginous and teleost fishes. The diverse strategies for responding to changes in salinity have led to differential regulation and tissue-specific effects of hormones. Combining traditional physiological approaches with genomic, transcriptomic, and proteomic analyses will elucidate the patterns and diversity of the endocrine control of euryhalinity.

1. INTRODUCTION

Euryhalinity is originally an ecological term meaning an ability to live in broad (*eurys*) salinity (*halinos*) environments. Therefore, euryhaline fishes are those inhabiting estuaries, where salinity changes regularly, and those migrating between rivers [freshwater (FW)] and seas [seawater (SW)] during their lifespan. Osmoregulator is a physiological term for an organism with the ability to maintain body fluid osmolality at a certain level (usually around one-third of SW) irrespective of environmental salinities. Therefore, euryhaline fishes are always osmoregulators or ionoregulators. The endocrine system plays a pivotal role in manifesting euryhalinity, as it mediates homeostatic regulation to maintain ionic and water balance of the internal milieu. Various hormones have been implicated in acclimation to diverse environmental salinities in fishes, and while these are often conveniently grouped as FW-acclimating or SW-acclimating hormones (McCormick, 2001; Takei and Loretz, 2006), it should be noted that their function may differ among species or that they may even have dual functions depending on their interaction with other systems. As fish live in water, osmoregulation in the hypoosmotic FW environment is achieved by limiting osmotic water influx across body surfaces and by excreting via the kidney excess water that unavoidably enters the body (Marshall and Grosell, 2006). Obligatory loss of ions from the gills and kidney is compensated for by accelerating ion uptake by the gills and intestine. By contrast, acclimation to a hyperosmotic SW environment is achieved by increasing water gain by drinking environmental SW and subsequent water absorption by the intestine to compensate for osmotic water loss from the body surfaces (Marshall and Grosell, 2006). Excess monovalent ions (Na^+ and Cl^-) that enter the body surfaces (mainly the gills) and the intestine are actively secreted by ionocytes (also called mitochondrion-rich cells or chloride cells) in the gills and opercular epithelia. Therefore, euryhaline fishes must change drinking rate and reverse

water and ion fluxes at these major osmoregulatory organs (gills, intestine, and kidney) when they encounter hypoosmotic and hyperosmotic media, and this ability is the key to euryhalinity. Details of these mechanisms can be found in Edwards and Marshall (2013), Chapter 1, this volume.

The degree of euryhalinity (salinity tolerance) often changes during the lifespan of fishes. Highly migratory diadromous fishes spawn either in FW (anadromous) or in SW (catadromous) and the tolerance may differ in early life stages compared with fish preparing for migration. Fish may also lose amphihalinity after the end of migration. Many migratory fishes experience drastic changes in body functions, for example smoltification (salmonids) or silvering (eels), before migration into completely the opposite osmotic environment (see McCormick, 2013, Chapter 5, this volume). Thus, the ontogenic change in euryhalinity during early life stages and during maturation is an important theme in studying euryhalinity.

Euryhaline fishes are often capable of surviving direct transfer from FW to SW or vice versa known as amphihalinity. In the acute phase of acclimation, the sympathetic nervous system responds immediately and usually changes drinking rate and blood supply to the osmoregulatory organs such as gills, intestine, and kidney to regulate water and ion fluxes (Marshall, 2003). The nervous system also activates the hormonal system, which consists of rapid- and slow-acting hormones, to cope with the changes in a coordinated fashion. Rapid-acting hormones are amine or oligopeptide hormones, which are secreted immediately (seconds to minutes) upon the environmental changes for the rapid acclimation (minutes to hours), and removed quickly from the circulation (McCormick and Bradshaw, 2006; Takei, 2008). The rapid-acting hormones also act on the brain to regulate drinking and on the peripheral osmoregulatory organs to change the activity of various transport molecules (transporters, channels, pumps, and cell adhesion molecules) that are already present in the transport epithelium. Importantly, the rapid-acting hormones usually stimulate the secretion of slow-acting hormones that are involved in chronic acclimation to a new osmotic environment. The slow-acting hormones induce reorganization of osmoregulatory organs by *de novo* synthesis of transport molecules on the epithelial cell membrane and intercellular junctions, and by proliferation and differentiation of stem cells or morphogenesis of cell types to reverse the direction of ion and water fluxes. They are hormones that are secreted slowly (hours to days) and stay in the circulation for longer acclimation to a new osmotic medium (days to weeks). The interaction among the sympathetic nervous system, and the rapid and slow hormonal systems is crucial for permitting euryhalinity in fish. It should be noted that the categorization of hormones into rapid- and slow-acting hormones cannot be

absolute and some hormones may fall into both categories, as discussed below.

This chapter will review the hormonal regulation and induced mechanisms that permit reversal of ion and water regulation associated with euryhalinity. The chapter will be divided into sections that examine the role of rapid- and slow-acting hormones in controlling osmoregulation (Sections 2 and 3), the target organs for these hormones (from brain to kidney, Section 4), ontogeny (from eggs to adults, Section 5), and phylogeny (from cyclostomes to teleosts, Section 6), and the information will be integrated in relation to euryhalinity. The authors will attempt a complete summary and overview of the current state of knowledge and point out areas in need of more research. It should be noted that given the large number of fish species that exist and the relatively few that have been studied to date, these generalizations may not necessarily apply to all species.

2. RAPID-ACTING HORMONES

To cope with the sudden changes in environmental salinity, euryhaline fishes immediately shut off active transport machinery and activate existing transporters that often reverse the direction of ion and water transport via the

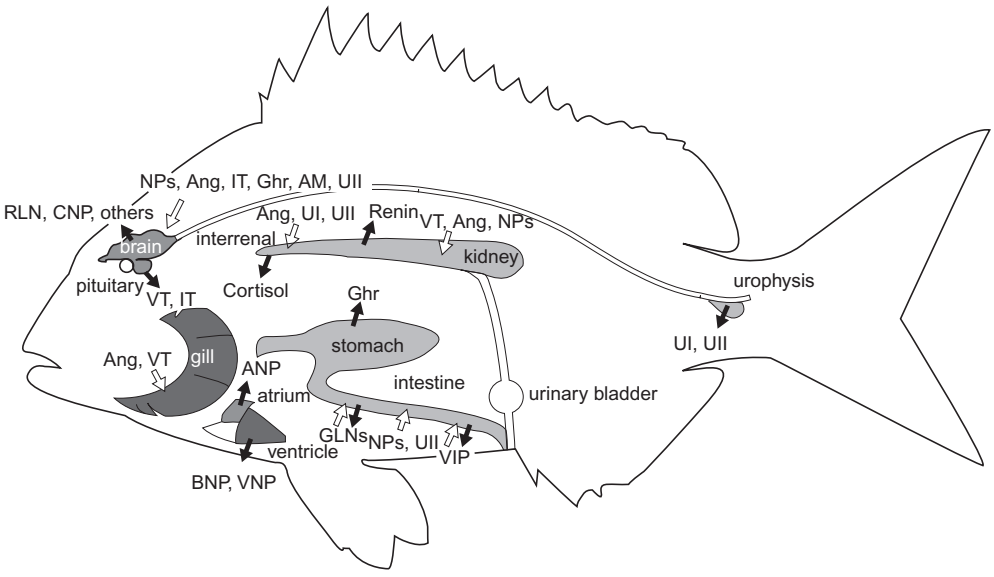

Fig. 3.1. Schema showing secretion (closed arrow) and action (open arrow) of rapid-acting hormones in teleost fishes. Ang: angiotensin II; ANP: atrial natriuretic peptide; AM: adrenomedullin; BNP: B-type natriuretic peptide; CNP: C-type natriuretic peptide; Ghr: ghrelin; GLN: guanylin; IT: isotocin; NP: natriuretic peptide; RLN: relaxin; VIP: vasoactive intestinal peptide; UI: urotensin I; UII: urotensin II.

nervous and endocrine systems (Marshall, 2003; Wood, 2011). In this acute phase, rapid- or short-acting hormones such as angiotensins, natriuretic peptides, neurohypophysial hormones, urotensins, and guanylins play a major role (McCormick, 2001; Takei, 2008; see Takei and Loretz, 2006, for details on the structure of these hormones). These oligopeptide hormones are secreted from the endocrine organs or produced in plasma by enzyme actions (renin–angiotensin system and kallikrein–kinin system) soon after encountering changes in environmental salinity. However, these hormones are metabolized by various peptidases in plasma or those associated with endothelial cells and disappear quickly from the circulation. Thus, the rapid-acting hormones take the role of combining the actions of the sympathetic nervous system and the slow-acting hormones. The tissues of synthesis/secretion and of action of rapid-acting hormones are summarized in Fig. 3.1.

Since plasma levels of some rapid-acting hormones increase before changes in plasma osmolality and blood volume occur, there must be an external sensor that detects the change in environmental osmolality or ion concentrations (see Kültz, 2013, Chapter 2, this volume), which transmits this information to the sympathetic nervous system and to the endocrine organs. Several proteins have been suggested to have osmosensing and/or Na^+-sensing functions, including adenylyl cyclase G (Saran and Schaap, 2004), transient receptor potential vallinoid type (TRPV) such as TRPV1, 2, and 4 (Liedtke and Kim, 2005), Nax channels (Shimizu et al., 2007), Ca-sensing receptor (Quinn et al., 1998) and aquaporin 4 (Venero et al., 1999). Osmosensing proteins that have been identified in fish (Fiol and Kültz, 2007) include Ca-sensing receptor in the dogfish (*Squalus acanthias*) (Nearing et al., 2002), gilthead seabream (*Sparus aurata*) (Flanagan et al., 2002), and Mozambique tilapia (*Oreochromis mossambicus*) (Loretz et al., 2004), and TRPV4 in sea bass (*Dicentrarchus labrax*) (Bossus et al., 2011) and Mozambique tilapia (Seale et al., 2011).

It is of interest to note that vasodepressor and natriferic (Na^+-extruding) hormones are much more diversified in fishes than in tetrapods, possibly to facilitate the complex osmoregulatory demands of aquatic life, as discussed in detail by Takei et al. (2007). For instance, vasodilatory (vasodepressor) hormones change blood flow to the epithelial cells of osmoregulatory organs (gills, intestine, and kidney) to regulate water and ion fluxes. Owing to their existence in water where they are much less influenced by the effects of gravity, fish have evolved low arterial pressure which may require dominant control by vasodepressor hormones. The reason for the diversified natriferic hormones is not clear as fishes in FW must retain Na^+ for body fluid homeostasis, but it may be related to the radiation of teleost fishes after they re-entered the marine environment around 160 million years ago (Nelson, 2006).

2.1. Angiotensins

The renin–angiotensin system (RAS) is an important hormonal system for the maintenance of water and ion balance in vertebrates (Kobayashi and Takei, 1996). Renin is a highly specific aspartyl proteinase that is secreted from the juxtaglomerular cells of the kidney and acts on angiotensinogen in plasma to cleave off an N-terminal decapeptide angiotensin I (Ang I). Subsequently, angiotensin-converting enzyme (ACE), a dipeptidyl carboxypeptidase, removes a C-terminal dipeptide from Ang I during the passage in the gill circulation to form the biologically active Ang II. Tetrapods have [Asp^1] Ang II (except for a highly aquatic clawed toad, *Xenopus laevis*), but all fishes have [Asn^1] Ang II, including lungfishes that are ancestral to tetrapods (Takei et al., 2004b). In recent years, the molecular and functional characterization of the RAS has rapidly expanded (Fyhrquist and Saijonmaa, 2008); not only N-terminal truncated Ang III [Ang II (2–8)] and Ang IV [Ang II (3–8)], but also C-terminal truncated Ang-(1–7) have been shown to have a range of biological actions. The second converting enzyme (ACE2) has been identified, which is a critical enzyme for Ang-(1–7) production (Xu et al., 2011). In addition to the new Ang peptides, new RAS receptors have been discovered (Fyhrquist and Saijonmaa, 2008). Previously, most biological actions of Ang II and Ang III were thought to be mediated by the AT1 and AT2 receptors, but it is now known that there is a Mas receptor for Ang-(1–7) (Xu et al., 2011), an insulin-regulated aminopeptidase receptor for Ang IV (Albiston et al., 2001), and a prorenin/renin receptor (Nguyen et al., 2002). Receptors for Ang IV and Ang-(1–7) mediate the specific actions of each ligand, and the prorenin/renin receptor appears to activate prorenin to form Ang II on the cell surface.

The pattern of changes in plasma osmolality after FW to SW transfer differs greatly among euryhaline species; plasma osmolality increases gradually with a peak after 1–2 days in eels (*Anguilla japonica*) (Okawara et al., 1987; Takei et al., 1998), but a peak in a few hours in Mozambique tilapia (Breves et al., 2010b) and striped bass (*Morone saxatilis*) (Tipsmark et al., 2007). As eels can readily survive direct transfer from FW to SW, there may be differences in the ability to tolerate acute salinity changes among euryhaline species in which rapid-acting hormones play critical roles. Plasma Ang II concentration increases transiently when eels are transferred from FW to SW, and the pattern of the increase exactly parallels that of plasma NaCl concentration (Okawara et al., 1987; Tierney et al., 1995; Wong and Takei, 2012). As NaCl loading inhibits renin secretion in mammals and birds (Takei et al., 1988a), this inhibitory mechanism may be absent in eels. Consistently, injection of hypertonic NaCl solution into the

circulation profoundly increased plasma Ang II concentration in eels (Takei et al., 1988b). It is now known that increased Cl^- excretion by the kidney is sensed by the macula densa of distal tubule to inhibit renin release in tetrapods (Kobayashi and Takei, 1996), but the macula densa is absent in teleosts. The data confirm the role of macula densa in renin secretion from the comparative viewpoint.

After the initial increase following SW transfer, plasma Ang II levels usually return to those in FW after SW acclimation in euryhaline fishes (Kobayashi and Takei, 1996), but are higher in fish acclimated to double-strength SW (Wong et al., 2006; Wong and Takei, 2012), indicating the role of Ang II in hyperosmotic acclimation. Detailed analysis of immunoreactive Ang II revealed that eel plasma contains comparable amounts of [Asn^1] Ang II, [Asp^1] Ang II, Ang III and Ang IV (Wong and Takei, 2012). Asparaginase appears to convert native [Asn^1] Ang II to [Asp^1] Ang II in the liver and kidney. Thus, previous studies using radioimmunoassay for Ang II measured all these Ang peptides as Ang II. It is necessary to compare the biological activity of these Ang peptides in fish. In eels, [Asn^1] Ang II was two-fold more potent than [Asp^1] Ang II and Ang III was much less potent for the vasopressor activity (Takei et al., 2004b).

When injected into the circulation, Ang II has a biphasic action on drinking in eels, causing an initial burst of drinking followed by prolonged inhibition (Takei et al., 1979), while constant acceleration of drinking was induced after an intracranial injection (Nobata and Takei, 2011). After peripheral injection, Ang II lowered net sodium reabsorption in the eel kidney (Nishimura and Sawyer, 1976), indicating its important role in SW acclimation. Ang II infusion reduced the glomerular filtration rate (GFR) of individual nephrons in rainbow trout (*Oncorhynchus mykiss*) (Brown et al., 1980). Ang II stimulated the activity of Na^+/K^+-ATPase (NKA) in isolated gill cells and renal tissues of eels (Marsigliante et al., 2000b), but inhibited it in isolated enterocytes (Marsigliante et al., 2001). The inhibition was mediated by a transient increase in intracellular calcium and subsequent protein kinase C activation. Furthermore, Ang II induced cortisol secretion *in vivo* and *in vitro* in several teleost species (Perrott and Balment, 1990). As cortisol is known to increase NKA abundance in most euryhaline fishes, Ang II appears to be involved indirectly in the chronic acclimation to SW (see Section 3.3, this chapter).

2.2. Natriuretic Peptides

The natriuretic peptide (NP) family consists of atrial, B-type, and ventricular natriuretic peptide (ANP, BNP, and VNP) secreted from the heart, and four C-type natriuretic peptides (CNP1–4) synthesized principally

in the brain (Toop and Donald, 2004). Comparative genomic analyses have indicated that CNP4 is an ancestral molecule of the NP family, from which CNP3 was first duplicated (Inoue et al., 2003). In the cyclostomes, only CNP4 is present and it is synthesized in both heart and brain (Kawakoshi et al., 2006). In elasmobranchs, however, CNP4 does not exist and only CNP3 is synthesized and secreted from the heart and brain (Kawakoshi et al., 2001). The cardiac ANP, BNP, and VNP were generated by tandem duplication from CNP3 on the same chromosome, while CNP1 and CNP2 were produced by block duplication from CNP3 (Inoue et al., 2003). It is apparent that all seven NPs existed when ray-finned and lobe-finned bony fishes diverged, as all types of NPs are present in the extant tetrapods with some deletions in different classes. For instance, ANP, BNP, and CNP4 are present in mammals, BNP, VNP, CNP1, and CNP3 in birds, ANP, BNP, CNP3, and CNP4 in amphibians, and ANP, BNP, VNP, and CNP1–4 in teleosts, with some exceptions. Although ray-finned fishes have the most diversified NPs among vertebrates, VNP is present only in some migratory teleosts such as eels and salmonids and in more basal ray-finned fishes such as bichir (*Polypterus endlicheri*) and sturgeon (*Acipenser transmontanus*). However, most ray-finned fishes have four CNPs, which indicates their important functions in this fish group.

Four types of NP receptors have been identified in eels. Whereas NPR-A (GC-A) and NPR-B (GC-B) have guanylyl cyclase in the cytoplasmic domain and use cyclic guanosine monophosphate cGMP as a second messenger, NPR-C and NPR-D have only a short cytoplasmic domain, and their function is thought to be ligand clearance, adjusting the concentration of NPs at various target tissues. However, new functions of NPR-C have been suggested via inhibition of cyclic adenosine 3′,5′-monophosphate (cAMP) production or stimulation of phospholipase C (Anand-Srivastava, 2005). Cardiac ANP and VNP bind NPR-A with high affinity, CNP1 binds NPR-B specifically, and all NPs bind NPR-C and NPR-D with high affinities in eels (Hirose et al., 2001). In medaka (*Oryzias latipes*), two types of NPR-A (OLGC2 and 7) and a single NPR-B (OLGC1) have been identified (Yamagami and Suzuki, 2005). Medaka has only BNP as a cardiac NP and four CNPs (Inoue et al., 2003). CNP1, 2, and 4 bind to OLGC1, CNP3 binds to OLGC2, and BNP binds to OLGC7 with high affinities (Inoue et al., 2003, 2005). Of note is the high affinity of CNP3, a direct ancestor of cardiac NPs, to an NPR-A type receptor.

Using a specific radioimmunoassay, it was shown that plasma ANP concentration increased transiently after transfer of eels from FW to SW and then returned to an FW level (Kaiya and Takei, 1996a). In mammals, hypervolemia is a primary stimulus for ANP secretion (Toop and Donald, 2004), but hypovolemia occurs after SW transfer in teleost fishes. It was

shown that an increase in plasma osmolality is the primary stimulus for ANP secretion in eels (Kaiya and Takei, 1996b). In rainbow trout, however, hypervolemia appears to be a potent stimulus for ANP secretion as in mammals (Cousins and Farrell, 1996). Research on more species is needed to determine whether such a difference is due to the particular adaptive strategy, which may differ among species.

Accumulating evidence indicates that ANP limits NaCl entry from the environment and facilitates SW acclimation. It was shown that ANP strongly inhibits drinking even at a non-depressor, physiological dose in eels (Tsuchida and Takei, 1998). Following SW transfer, there is an initial burst of drinking, but a transient suppression follows before the normal high drinking rate in SW fishes is re-established. The transient inhibition of drinking mirrors the transient increase in plasma ANP concentration after SW transfer (Kaiya and Takei, 1996a). Thus, ANP may delay an immediate increase in plasma osmolality that would otherwise occur, thereby promoting the initial phase of SW acclimation. Furthermore, ANP is also involved in the chronic inhibition of excess drinking because removal of circulating ANP by immunoneutralization enhanced the drinking rate and plasma Na^+ concentration in SW acclimated eels (Tsukada and Takei, 2006). As mentioned above, injection of hypertonic solutions, which is the most potent dipsogenic stimulus in tetrapods (Fitzsimons, 1998), clearly suppressed drinking in SW eels (Takei et al., 1988b). This may be due to the profound ANP secretion after hyperosmotic stimulus (Kaiya and Takei, 1996b). These results show that ANP is a major regulator of drinking in eels and probably in other teleost species. Recently, the potency order was shown to be $ANP = VNP > BNP = CNP3 >> CNP1 = CNP4$ in eels (Miyanishi et al., 2011). CNP3 exhibited a stronger antidipsogenic effect than other CNPs.

ANP inhibits intestinal NaCl absorption to further limit NaCl entry into the body in a few teleost species (O'Grady et al., 1985; Ando et al., 1992; Loretz et al., 1997). The inhibitory effect is highly potent at physiological concentrations (two to three orders more potent than other hormones) and highly efficacious (nullified short-circuit current) at high concentrations. Thus, it is most likely that ANP is a physiological regulator of intestinal NaCl absorption. The effect is mediated by the inhibition of $Na^+/K^+/2Cl^-$ cotransporter type 2 (NKCC2), a major transporter that facilitates water absorption (O'Grady et al., 1985). Thus, water absorption may be inhibited by ANP, which in the long term is disadvantageous for SW acclimation. This may reflect the fact that eels primarily regulate plasma osmolality over blood volume for body fluid regulation (Takei and Balment, 2009).

Concerning NaCl excretion, ANP increases urine Na^+ concentration but reduces urine volume in SW eels, resulting in a constant rate of total NaCl

excretion by the kidney (Takei and Kaiya, 1998). This effect may be unique in eels because ANP induces profound natriuresis and diuresis in the trout (Duff and Olson, 1986). It was shown that 99.5% of NaCl is excreted via the gills in SW eels, but ANP failed to increase ^{22}Na excretion into the medium, indicating no direct action of ANP on the branchial Na^+ excretion (Tsukada et al., 2005). However, ANP may indirectly stimulate the branchial NaCl excretion via secretion of slow-acting hormones. It has been shown that ANP stimulates cortisol secretion *in vivo* in the flounder and *in vitro* from the interrenal tissue of trout (Arnold-Reed and Balment, 1991). In the eel, ANP also stimulates cortisol secretion *in vivo* (Li and Takei, 2003), but it failed to stimulate cortisol secretion *in vitro* from the interrenal tissue (Ventura et al., 2011). It is now found that ANP was effective *in vivo* in eels as it enhances the steroidogenic action of adrenocorticotropic hormone (ACTH). The steroidogenic effect was observed only in SW-acclimated eels. ANP also stimulates growth hormone (GH) secretion from the dispersed pituitary cells of Mozambique tilapia (Fox et al., 2007). Since cortisol and GH work in concert to promote differentiation of SW-type ionocytes in salmonid fishes (Madsen, 1990; McCormick, 2001), ANP can increase branchial NaCl secretion through slow-acting hormones in the late stage of SW acclimation.

In contrast to ANP, CNP appears to be an FW-acclimating hormone (Takei and Hirose, 2002). CNP was first isolated from the brain of killifish and eel, and as the CNP sequence was almost identical to mammalian CNP, this was initially thought to be a teleostean homologue of mammalian CNP. However, comparative genomic analyses later showed that the first teleostean CNP is CNP1 and mammalian CNP is an orthologue of teleostean CNP4 (Inoue et al., 2003). Plasma CNP1 concentration measured by radioimmunoassay for eel CNP1 was higher in FW eels than in SW eels (Takei et al., 2001). However, as CNP3 and CNP4 were later identified in the eel (Nobata et al., 2010), it is possible that the radioimmunoassay measured these CNPs also as CNP1. On the other hand, CNP1 infusion increased ^{22}Na uptake from the environment and increased plasma Na^+ concentration in FW eels (Takei and Hirose, 2002). In dispersed pituitary cells of Mozambique tilapia, CNP1 has no effect on prolactin secretion (Fox et al., 2007). As CNP3 is expressed abundantly in the pituitary (Nobata et al., 2010), the effect of CNP3 on prolactin secretion for FW acclimation needs to be examined.

2.3. Guanylins

Guanylin has a dual identity as an endocrine hormone and exocrine ectohormone because it is secreted into the intestinal lumen that is outside the internal milieu. The guanylin family is also diversified in teleost fishes. Three guanylins have been identified in the eel; guanylin is synthesized only

in the intestine, while uroguanylin and renoguanylin are produced also in other segments of the digestive tracts and the kidney (Yuge et al., 2003; Kaljnaia et al., 2009). Guanylin is synthesized by the goblet cells of the eel intestine and secreted into the lumen with mucus (Yuge et al., 2003), and uroguanylin may be synthesized by the enterochromaffin cells and secreted in both directions (lumen and circulation) as in mammals (Nakazato, 2001). Uroguanylin is resistant against degrading enzymes on the brush-border membrane of renal proximal tubules, but renoguanylin may be metabolized quickly if it is secreted into the lumen of renal tubules. Therefore, uroguanylin may act on renal tubules from the luminal side and the final urine contains significant amounts of uroguanylin in teleost fishes as in mammals (Forte et al., 2000). Two types of guanylin receptors have been identified in eels, GC-C1 and GC-C2 (so named as they have a GC domain intracellularly), and uroguanylin has higher affinity to GC-C1 while guanylin and renoguanylin have higher affinities to GC-C2 (Yuge et al., 2006). This coincides with the higher expression of the GC-C1 gene in the kidney. The affinity of guanylin to GC-C is lower than that of NPs to GC-A and GC-B (NPR-A and NPR-B), probably because NPs are secreted into the circulation and guanylins into the intestinal lumen.

None of the hormones discussed so far as being involved in SW acclimation shows changes in messenger RNA (mRNA) expression in response to SW transfer. For instance, the ANP mRNA levels were not significantly increased after transfer of eels from FW to SW (H. Kaiya, unpublished data). However, intestinal guanylin mRNA expression increased five-fold 24 h following SW transfer relative to levels in FW-acclimated eels (Yuge et al., 2003). Furthermore, the expression of GC-C1 and GC-C2 genes was also upregulated in the intestine of SW eels compared to FW eels (Yuge et al., 2006). The higher expression of both hormone and receptor genes in acute and chronic phases of SW acclimation strongly suggests their important roles in SW acclimation.

Like cardiac ANP and VNP, guanylins inhibited short-circuit current in a dose-dependent manner when applied to the luminal (mucosal) side of intestinal epithelia and reversed the current at high doses (Yuge and Takei, 2007). It was shown that the reversal is due to Cl^- secretion via the cystic fibrosis transmembrane conductance regulator (CFTR)-type Cl^- channel as in mammals. Interpretation of these observations has been outlined by Takei and Yuge (2007) as follows. After imbibed SW is desalted in the esophagus, which is highly permeable to NaCl, but not water (Hirano and Mayer-Gostan, 1976), and further diluted to isotonicity in the stomach, water is absorbed together with monovalent ions in the anterior intestine from the isotonic luminal fluid. The major transporter for ion absorption is NKCC2 on the apical membrane of absorptive epithelial cells. However, as SW contains

similar concentrations of Na^+ and Cl^-, Cl^- becomes deficient in the luminal fluid after absorption of one Na^+ and two Cl^- by NKCC2, which depresses NKCC operation and, thus, decreases water absorption. In fact, there seems to be Cl^- secretion into the lumen as judged by the maintained luminal Cl^- concentration along the intestine compared with Na^+ (Tsukada and Takei, 2006). It is possible that guanylin is involved in the active Cl^- secretion into the lumen to supplement luminal Cl^- to ensure water absorption in the posterior intestine. It has also been shown that guanylin stimulates HCO_3^- secretion through CFTR to precipitate concentrated Ca^{2+} and Mg^{2+} ions after water absorption (M. Ando and Y. Takei, unpublished data). There is accumulating evidence showing that active secretion of HCO_3^- into the lumen precipitates $Mg/CaCO_3$ and decreases luminal fluid osmolality, thereby facilitating water absorption (Grosell et al., 2009; Wilson et al., 2009). It remains to be determined whether guanylins are secreted in response to high luminal NaCl concentration or osmolality of imbibed SW as reported in mammals (Nakazato, 2001), and whether guanylin actually promotes SW acclimation by secretion of Cl^- and HCO_3^- into the lumen.

2.4. Neurohypophysial Hormones

Neurohypophysial hormones, vasotocin (VT) and isotocin (IT), are likely to be important osmoregulatory hormones in teleost fishes, as vasopressin, an orthologue of VT, is critical for body fluid regulation in terrestrial animals through its effect on tubular water reabsorption in the kidney (Babey et al., 2011). A recent *in silico* study has shown that teleosts possess at least five distinct receptors [two V1-type receptors (V1R), two V2-type receptors (V2R) and an oxytocin-type receptor] for VT and IT (Daza et al., 2012). A V2R of medaka and bichir (*P. senegalus*) transiently expressed in culture cells responded to VT with cAMP accumulation, consistent with what has been observed with the mammalian V2R (Konno et al., 2010). As mentioned in detail below (Section 4.3), urine volume is primarily regulated by GFR in teleost fishes and the nephron is unable to concentrate urine above plasma because of the lack of a countercurrent system formed by the loop of Henle (Nishimura and Fan, 2003). Therefore, the tubular effect of VT for water reabsorption may be minor in teleost fishes.

VT may be involved in acclimation to high-salinity environments, but the data are still somewhat controversial. An initial study suggested the involvement of VT in FW acclimation as the VT mRNA levels decreased for 2 weeks after transfer of rainbow trout from FW to 80% SW and increased again following transfer back to FW (Hyodo and Urano, 1991). The IT gene did not exhibit obvious changes after either transfer. However,

hypothalamic VT mRNA levels increased 4 h after transfer of euryhaline flounder (*Platichthys flesus*) from FW to SW, with concomitant increases in plasma VT concentration (Warne et al., 2005). Plasma VT concentration decreased after transfer of the flounder from SW to FW (Bond et al., 2002). In primary culture of sea bass gill pavement cells grown on a permeable support, VT and IT decreased short-circuit current (Cl^- secretion) in a dose-dependent manner with IT more potent and efficacious than VT (Guibbolini and Avella, 2003). V1R antagonist blocked the VT effect, but V2R agonist and antagonist had no effect, suggesting mediation by V1R. Chronic VT treatment increased gill NKA activity and plasma cortisol levels in the gilthead sea bream (Sangiago-Alvarellos et al., 2006). VT at physiological concentrations caused a dose-dependent reduction in urine flow and reduced filtering population of glomeruli to one-third in the *in situ* perfused trout kidney (Amer and Brown, 1995). In eels, IT stimulated drinking through its relaxing effect on the esophageal sphincter muscle while VT inhibited drinking, antagonizing the IT effect (Ando et al., 2000; Watanabe et al., 2007).

2.5. Urotensins

Urotensins (UI and UII) were first expected to have osmoregulatory functions in fishes because they are secreted from the urophysis, which is upstream and in direct circulatory contact with the kidney and intestine (McCrohan et al., 2007). U1, the orthologue of which in mammals was named urocortin, is a member of the corticotropin-releasing hormone (CRH) family and thus stimulates ACTH secretion, resulting in cortisol secretion (Lovejoy and Balment, 1999). UI (urocortin) and CRH bind to CRH type 1 (CRHR1) and type 2 (CRHR2) receptors. The CRHR1 has similar affinities to both ligands, whereas the CRHR2 exhibits higher affinity to UI. The goby (*Gillichthys mirabilis*), transferred from SW to FW, increased urophyseal UI content after 24 h, showing osmotic sensitivity of the urophysis (Larson and Madani, 1991). Initial physiological studies reported that UI inhibited water and NaCl absorption in isolated anterior intestinal segments of FW-acclimated, but not SW-acclimated, Mozambique tilapia (Mainoya and Bern, 1982). In the flounder, UI appears to stimulate cortisol secretion directly and interact synergistically with ACTH (Kelsall and Balment, 1998). Given cortisol's role in both FW and SW acclimation (see Section 3.3, this chapter), these results implicate a possible role of UI in FW acclimation.

UII is a member of the somatostatin superfamily and thus likely to be involved in the inhibition of GH secretion (Tostivint et al., 2008). Expression of the UII gene and its receptor (UT) gene was reduced after

acute transfer of euryhaline flounder from SW to FW, and the UT gene expression in the gills and kidney is downregulated in FW-acclimated fish, although plasma UII levels do not differ between SW and FW fishes (Lu et al., 2006). However, plasma UII concentration was reduced for some time after transfer of the flounder from SW to FW, showing the rapid-acting nature of UII (Bond et al., 2002). Initial physiological studies revealed that UII has a direct action on the intestine to increase water and NaCl absorption in SW-acclimated tilapia (Mainoya and Bern, 1982) and 5% SW-acclimated goby (Loretz et al., 1983) and on the urinary bladder of SW-acclimated goby (Loretz and Bern, 1981). UII inhibited prolactin secretion from the rostral part of Mozambique tilapia pituitary (Grau et al., 1982). These results suggest a role of UII in SW acclimation. However, UII inhibited short-circuit current in the goby skin, suggesting an inhibition of active Cl^- secretion, probably via ionocytes (Marshall and Bern, 1981). As UI reversed the effect of UII in the goby skin, UI and UII may be involved in the acclimation to opposite osmotic environments. More recently, UII was found to potently inhibit drinking in eels through its action on the brain (Nobata et al., 2011). UII stimulated cortisol secretion from the interrenal cells of rainbow trout (Arnold-Reed and Balment, 1994).

2.6. Adrenomedullins

Adrenomedullin (AM) is a member of the calcitonin gene-related peptide (CGRP) family that consists of CGRP, AM, and amylin (López and Martínez, 2002). However, five AMs (AM1–5) form a subfamily in teleost fishes, of which AM in mammals is an orthologue of teleost AM1 (Ogoshi et al., 2003). This finding in teleosts led to the discovery of AM2 and AM5 in mammals (Takei et al., 2004a, 2008; Ogoshi et al., 2006). Accordingly, it is now generally accepted that the CGRP family is comprised of CGRP, AM, AM2, AM5, and amylin in fishes and tetrapods, and AM4 and AM3 are generated from AM1 and AM2, respectively, by the whole-genome duplication that occurred only in the teleost lineage (Ogoshi et al., 2006). AM1 binds calcitonin receptor-like receptor (CLR) associated with receptor activity-modifying protein (RAMP) 2 or 3, and uses cAMP as a second messenger (Hay et al., 2005). AM2 and AM5 bind to CLR and RAMP3 complex with low affinity, and thus the specific receptor to these peptides may be present. Judging from the retained multiple paralogues after genome duplication and potent vasodepressor and natriferic actions in eels (Nobata et al., 2008; Ogoshi et al., 2008), the AM peptides may have important functions for SW acclimation as observed with the NP peptides.

The tissue distribution of the AM mRNA differs among teleost species. In tiger pufferfish (*Takifugu rubripes*), AM1/4 is ubiquitously expressed in various tissues, AM2/3 mostly in the brain, and AM5 in the spleen and gills (Ogoshi et al., 2003). In the eel, AM1 is expressed in the heart, kidney, and red body of air bladder, AM2/3 in a large number of tissues, and AM5 in the spleen and red body (Nobata et al., 2008). The expression of the AM genes in the major osmoregulatory organs did not change after transfer of pufferfish from SW to FW (M. Ogoshi and Y. Takei, unpublished results) or between FW- and SW-acclimated eels (Nobata et al., 2008). AMs are highly efficacious vasodepressor hormones in eels with a potency order of AM2 > AM5 > > AM1 (Nobata et al., 2008), which is different from the results in mammals where the effects of AM1 and AM2 are comparable (Takei et al., 2004a). Concerning their renal action, AM2 and AM5 infused into the circulation of FW eels caused antidiuresis without changes in blood pressure but AM1 infusion caused antinatriuresis (Ogoshi et al., 2008). AM2 and AM5 induced drinking as potently as Ang II when infused into the circulation of FW eels, but intracranial injection failed to affect drinking, although Ang II was effective when injected into the same site. Further investigations are necessary to define the role of AMs in fish osmoregulation.

2.7. Other Peptide Hormones

Relaxins (RLN1/2 and 3) belong to the insulin superfamily that contains insulin, insulin-like peptides, and insulin-like growth factors (Wilkinson et al., 2005). Three RLNs were identified in humans, but RLN1 and 2 were generated by tandem duplication only in primates. Thus, two RLNs generally exist in mammals, of which RLN1/2 is principally a peripheral hormone involved in cardiovascular regulation and reproduction, while RLN3 is a neuropeptide whose function has not yet been fully elucidated. In teleost fishes, three relaxins (RLN1/2, RLN3a, and RLN3b) are present in all species thus far examined (Good-Avila et al., 2009). All three teleost RLNs are produced in the brain of eels and the sequences are highly conserved even between RLN1/2 and RLN3 (Hu et al., 2011). RLNs may be involved in body fluid regulation since RLN1/2 was shown to have a potent dipsogenic effect in mammals (Sunn et al., 2002). However, expression of the three RLN genes does not differ between FW and SW eels (Hu et al., 2011). More research is needed to evaluate the role of RLNs in osmoregulation in teleost fishes.

Vasoactive intestinal peptide (VIP) is a member of the secretin superfamily and a close paralogue of pituitary adenylate cyclase-activating peptide (PACAP) (Sherwood et al., 2000). VIP and PACAP share the PAC1 and VPAC receptors and act through stimulation of intracellular cAMP (Cardoso et al., 2007). VIP is duplicated in some teleost species, while two PACAP peptides exist in all species thus far examined (Takei, 2008). VIP has long been known to affect osmoregulation, where it has been shown to stimulate Cl^- secretion from the opercular epithelia of SW-acclimated Mozambique tilapia (Foskett et al., 1982). Furthermore, VIP was shown to inhibit the short-circuit current (NaCl absorption) in the tilapia intestine (Mainoya and Bern, 1984) and in the SW eel intestine (Ando et al., 2003). In the intestine of winter flounder, VIP stimulated Cl^- secretion through cAMP acting as a second messenger (O'Grady and Wolters, 1990). More recently, VIP was found to have no effect on drinking when injected centrally and peripherally in the eel, although it is dipsogenic in the rat after central injection (Ando et al., 2003). VIP and PACAP regulate the secretion of pituitary hormones, renin, and adrenal steroids in mammals (Sherwood et al., 2000; Vaudry et al., 2009), but in teleost fishes only PACAP has been implicated in GH release (Canosa et al., 2007).

3. SLOW-ACTING HORMONES

Hormones such as prolactin, GH/insulin-like growth factor-1 (IGF-I), and cortisol act to alter the overall capacity for osmoregulation in teleost fishes. These protein hormones and steroid hormones have relatively long half-lives in plasma and reorganize osmoregulatory organs primarily by *de novo* synthesis of transport proteins, transporter/channel/pumps, and intercellular matrix, and through regulation of cell proliferation and differentiation.

3.1. Prolactin

Fifty years ago, Grace Pickford found that removal of the pituitary resulted in mortality in FW, but not in SW, and that survival in FW could be restored by treatment with prolactin in the killifish (*Fundulus heteroclitus*) (Pickford and Phillips, 1959). Since this classic study, a great deal of evidence has been generated supporting the involvement of prolactin in the process of FW acclimation in teleost fishes. This evidence includes changes in prolactin gene expression and circulating levels in response to salinity change, localization and regulation of prolactin receptors, and further

studies on the osmoregulatory mechanisms induced with prolactin treatment (see reviews by McCormick, 2001; Manzon, 2002; Sakamoto and McCormick, 2006).

For most euryhaline teleosts, gene transcription, synthesis, secretion, and plasma levels of prolactin all increase following exposure to FW (Manzon, 2002; Lee et al., 2006). Stenohaline FW fishes also appear to adjust prolactin production in response to low ion concentrations (Liu et al., 2006; Hoshijima and Hirose, 2007), even at very early developmental stages. In some euryhaline fishes lactotrophs are directly responsive to osmolality (Seale et al., 2002), although this is not universal (Kelley et al., 1990). Cortisol has a negative effect on prolactin secretion that can be both rapid and sustained (Kelley et al., 1990; Borski et al., 2002). Metabolic clearance rates of prolactin in salmonids are also increased following FW acclimation (Sakamoto et al., 1991), suggesting increased utilization, metabolism, and/or excretion. As in mammals, prolactin-releasing peptide (PrRP) has been identified in teleost hypothalamus; this peptide increases prolactin secretion and is expressed at higher levels in FW than in SW (Moriyama et al., 2002; Sakamoto et al., 2005).

Prolactin receptor transcription and abundance are high in osmoregulatory organs such as the gill, intestine, and kidney, and are normally in greater abundance in FW than SW (Fryer, 1979; Dauder et al., 1990; Auperin et al., 1995). High levels of prolactin receptor transcription have been found in gill ionocytes and enterocytes, cells specifically involved in osmoregulation (Sandra et al., 2000). In zebrafish (*Danio rerio*), gene knockout experiments have shown that prolactin receptors are necessary for early development of pituitary function and the capacity to respond to low ion environments with prolactin transcription (Liu et al., 2006). Multiple prolactin receptor isoforms have been described in several species and are likely to differ in their physiological functions, although the nature of these differences has yet to be determined. Expression of tilapia prolactin receptor (PRLR) 1 and 2 in a heterologous expression system indicates that the receptors activate different downstream signaling pathways with different actions on cell ion regulatory capacity (Fiol et al., 2009). Furthermore, PRLR isoforms in zebrafish and tilapia are tissue specific in their expression and respond differentially to changes in environmental ion concentrations (Breves et al., 2011; J. P. Breves, personal communication).

The response of fish in FW to removal of the pituitary is not universal. Some FW species such as rainbow trout and goldfish are able to survive in FW for sustained periods following hypophysectomy. Amphihaline eels (*Anguilla* sp.) spend the majority of their life cycle in FW and can survive in FW after hypophysectomy (Hirano, 1969). In the case of goldfish (*Carassius*

auratus), hypophysectomy results in lower levels of plasma ions that can be restored by prolactin. Amphihaline Mozambique tilapia die in FW after hypophysectomy, but can survive in SW (Breves et al., 2010b). When these patterns across species are considered together, it seems plausible that FW species have a level of constitutive (prolactin-independent) ion uptake capacity that may be absent in brackish water and marine species, and an additional prolactin-dependent regulation that allows for regulation in the face of increased demand for ion uptake such as ion poor and acidic conditions. The sea bream does not change circulating prolactin levels after exposure to FW, nor does the isolated pituitary alter prolactin secretion in response to changes in osmotic pressure (Fuentes et al., 2010), suggesting that prolactin may have a limited role in controlling osmoregulation in stenohaline marine species.

A large number of prolactin's actions are associated directly or indirectly with cell proliferation and/or apoptosis (Sakamoto and McCormick, 2006). Prolactin has been shown to affect ionocytes, both by inhibiting the development of secretory ionocytes (Herndon et al., 1991; Kelly et al., 1999) and by promoting the morphology and functional attributes of ion uptake cells (Pisam et al., 1993). Prolactin also regulates permeability characteristics of epithelial tissue (Manzon, 2002). For example, prolactin has been shown to reduce transcellular permeability, characteristic of exposure to FW, in an *in vitro* gill pavement cell culture system (Kelly and Wood, 2002b). More recent studies indicate that proteins involved in active ion transport by the gill are regulated by prolactin, including downregulation of the SW isoform of NKA (NKAα1b) (Tipsmark and Madsen, 2009), upregulation of the FW isoform NKAα1a (T. O. Nilsen, S. Stefansson, and S. D. McCormick, unpublished results), and gill NKA activity (Shrimpton and McCormick, 1998) in Atlantic salmon. In tilapia, gill mRNA levels of the apical Na^+/Cl^- cotransporter (NCC) and NKAα1a, both of which are involved in ion uptake, are reduced after hypophysectomy and restored by prolactin treatment (Breves et al., 2010c; Tipsmark et al., 2011). Intestinal claudins 15 and 25b that are upregulated by SW exposure are downregulated by prolactin treatment (Tipsmark et al., 2010b). Prolactin treatment also reduces ion and water permeability of the esophagus and intestine, a response that normally occurs during acclimation to FW, perhaps acting through regulation of cell apoptosis and proliferation (Takahashi et al., 2006).

There is some evidence for the interaction of prolactin and cortisol in promoting ion uptake, and it is possible that prolactin is acting as a "switch" for promoting ion uptake in the same way that the GH–IGF-I axis interacts with cortisol to promote salt secretion (McCormick, 2001). In hypophysectomized and/or interrenalectomized fish, prolactin and/or ACTH or cortisol are necessary to completely restore ion and water balance in FW (McCormick, 2001). In hypophysectomized channel catfish (*Ictalurus punctatus*), prolactin

and cortisol in combination cause a greater restoration of plasma ions than either acting alone (Eckert et al., 2001). Also, cortisol and prolactin together have a greater effect than either hormone alone on promoting the transepithelial resistance and potential and ion influx of an *in vitro* gill cell preparation from rainbow trout (Zhou et al., 2003). In hypophysectomized tilapia in FW, ovine prolactin and cortisol stimulated fxyd-11 expression, a subunit gene of NKA involved in modulation of its activity, in a synergistic manner (Tipsmark et al., 2011). These studies support an interaction between prolactin and cortisol in controlling ion uptake in fish, although the universality of this model is unclear, and there is little understanding of the cellular pathways that may be involved. Prolactin decreases transcription of liver IGF-I (Tipsmark and Madsen, 2009), providing a possible pathway for how prolactin may interfere with signals that promote salt secretion.

3.2. Growth Hormone/Insulin-Like Growth Factor-1

D. C. W. Smith (1956) observed that multiple injections of GH could increase the capacity of brown trout to tolerate exposure to SW. This was at first attributed to the growth effect of GH because size confers greater salinity tolerance in salmonids. More recently it was found that a single injection of GH in unfed fish was sufficient to increase salinity tolerance, indicating a relatively rapid effect that was independent of body size (Bolton et al., 1987). This effect of GH on salinity tolerance has been found in two other phylogenetically disparate euryhaline species, tilapia and killifish (Sakamoto et al., 1997; Mancera and McCormick, 1998).

A major route of the osmoregulatory action of GH is through its capacity to increase circulating levels and local tissue production of IGF-I. Exogenous treatment of IGF-I has been found to increase the salinity tolerance of rainbow trout, Atlantic salmon, and killifish (McCormick, 2001). GH cannot directly increase NKA activity in cultured gill tissues (McCormick and Bern, 1989), whereas IGF-I can (Madsen and Bern, 1993). The ability of prior GH treatment to increase *in vitro* responsiveness of gill tissue to IGF-I further suggests an indirect action of GH on gill tissue, and a direct action of IGF-I (Madsen and Bern, 1993).

Increased gene expression, secretion, circulating levels, and metabolic clearance rate of GH and IGF-I after exposure to SW provide strong evidence for their hypoosmoregulatory actions in salmonids (Sakamoto et al., 1990, 1993; Sakamoto and Hirano, 1993). Binding proteins may also play a role, as circulating levels of the 21, 42, and 50 kDa IGF-I binding proteins change after SW exposure of rainbow trout (Shepherd et al., 2005). *In vitro*, the tilapia pituitary responds to physiologically relevant elevations of extracellular osmolality with increased GH secretion (Seale et al., 2002),

and plasma GH levels have been found to increase after SW exposure (Yada et al., 1994; Breves et al., 2010a). Plasma GH levels also increase in stenohaline FW catfish following exposure to brackish water (Drennon et al., 2003). GH transcription has also been detected in osmoregulatory organs (Yang et al., 1999), opening up the possibility that GH is acting in an autocrine or a paracrine manner in these tissues. IGF-I mRNA levels in liver, gill, and kidney increase following GH injection and exposure to SW in rainbow trout (Sakamoto and Hirano, 1993). Similar responses were seen in the gill of tilapia with increased transcription of IGF-I, IGF-2, and GH receptor, whereas SW caused decreased transcription of these genes in the kidney (Link et al., 2010). IGF-I has been found at higher levels in gill ionocytes than in other cell types in the gill (Reinecke et al., 1997).

High levels of GH receptors as measured by GH binding have been found in the liver, gill, gut, and kidney of euryhaline fishes. The proportion of hepatic GH receptors bound by GH increases following exposure to SW (Sakamoto and Hirano, 1991). GH receptor transcription also has been detected at high levels in osmoregulatory organs, and in the gill is upregulated by environmental salinity in salmonids, Nile tilapia, and flounder (Kiilerich et al., 2007b; Nilsen et al., 2008; Meier et al., 2009; Breves et al., 2010b). Specific high-affinity, high-capacity IGF-I receptors have been found in gill tissue of salmon and tilapia (S. D. McCormick and A. Regish, unpublished results), and have been immunocytochemically localized to gill ionocytes in striped bass, where their transcription is increased by SW exposure (Tipsmark et al., 2007).

The GH–IGF-I and cortisol axes interact to regulate salt secretion in teleosts. Simultaneous treatment with GH and cortisol increases salinity tolerance and gill NKA activity in salmonids and killifish to a greater extent than either hormone alone (Madsen, 1990; Mancera and McCormick, 1998; McCormick, 2001). Cortisol treatment of Atlantic salmon in FW causes an increase in both the FW and SW isoforms of NKA, but treatment with GH and cortisol causes the SW isoform to increase to an even greater extent, and the FW isoform to decrease (S.D. McCormick, unpublished results). These findings suggest that GH is acting as a switch for the effects of cortisol, shifting its actions away from ion uptake and towards salt secretion (Fig. 3.2). At least some of the interaction of GH and cortisol is through GH's capacity to upregulate the number of gill cortisol receptors (Shrimpton and McCormick, 1998), which makes the tissue more responsive to cortisol. Cortisol also increases gill transcription of GH and IGF-I receptors in Atlantic salmon, providing another potential pathway for interaction (Tipsmark and Madsen, 2009).

GH–IGF-I has been shown to regulate several specific ion transporters and other proteins involved in osmoregulation in SW. It has been known for some time that GH and IGF-I can increase the activity of NKA in the

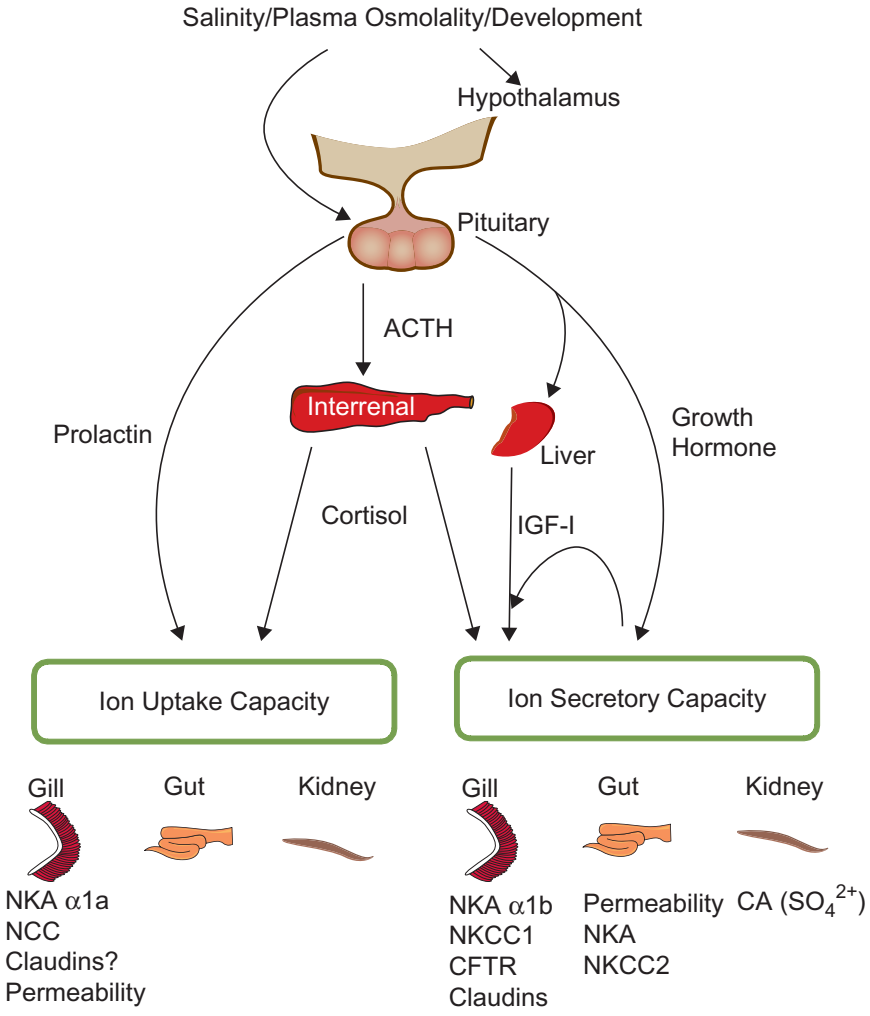

Fig. 3.2. Effect of slow-acting hormones on the ion uptake and salt secretory capacity of euryhaline fishes, emphasizing the interaction of prolactin and cortisol in promoting ion uptake and the interaction of the GH–IGF-I axes and cortisol in promoting salt secretion. The lower part of the figure shows proteins involved in osmoregulation that are known to be under the control of these endocrine systems for gill, gut, and kidney. PrP: prolactin-releasing peptide; GH: growth hormone; ACTH: adrenocorticotropic hormone; IGF-I: insulin-like growth factor-1; NKA: Na^+/K^+-ATPase; NCC: Na^+/Cl^- cotransporter; NKCC: $Na^+/K^+/2Cl^-$ cotransporter; CFTR: cystic fibrosis transmembrane regulator; CA = carbonic anhydrase.

gill, which also increases after SW acclimation in most teleosts (McCormick, 2001). More recently, the abundance and/or transcription of the three transport proteins most directly involved in salt secretion, NKAa1b, NKCC, and CFTR (see Edwards and Marshall, 2013, Chapter 1, this volume) have been found to be increased by the GH–IGF-I axis (Pelis and McCormick, 2001; Tipsmark and Madsen, 2009). These genes are also regulated by cortisol, which may reflect the important interaction

between these two endocrine axes. Since cortisol and IGF-I can upregulate many of these targets *in vitro*, it appears that cortisol-dependent and -independent pathways are present. GH also increases the gill transcription of FXYD-11 (Tipsmark et al., 2010a). In the gut, claudins 15 and 25b are upregulated by exposure to SW and GH treatment in FW, but these are inhibited by cortisol (Tipsmark et al., 2010b). These findings suggest that the positive interaction of the GH–IGF-I and cortisol axes that have been found for the gill may not be present in the gut and perhaps the kidney.

GH increases mitotic activity in several cell types in the gill of rainbow trout. Cortisol has no effect on mitotic activity but increases the number of ionocytes, suggesting that cortisol acts primarily to promote their differentiation. Therefore, another pathway for GH–IGF-I and cortisol interaction is stimulation of stem/progenitor cell proliferation by GH and/or IGF-I, creating more stem cells that can then be acted on by cortisol. The GH–IGF-I axis and cortisol may also interact at "higher" regulatory pathways, such as the hypothalamus and pituitary. *In vivo* and *in vitro* exposure to GH increases the sensitivity of interrenal tissue to ACTH, causing increased release of cortisol (Young, 1988). CRH is a potent stimulator of *in vitro* GH release in eels (Rousseau et al., 1999).

To date, a relatively small number of teleosts have been examined for the physiological impact of the GH–IGF-I axis on osmoregulation. Exogenous treatments have been found to affect most salmonids, Mozambique tilapia, and killifish. Convincing evidence for endocrine and paracrine actions of the GH–IGF-I axis comes from circulating hormones, local production, and from salmonids and tilapia, but there is relatively little information in this area from other teleosts, especially stenohaline species, which would offer a contrast to euryhaline models. However, there is no apparent effect of exogenous GH on several osmoregulatory parameters in the euryhaline gilthead sea bream (*Sparus aurata*) (Mancera et al., 2002), and osmoregulatory effects on euryhaline silver sea bream (*Sparus sarba*) are not consistent with an SW acclimating impact (Kelly et al., 1999). Pituitary GH and liver IGF-I mRNA levels in sea bream were lower after exposure to both hypersaline and hyposaline conditions (Deane and Woo, 2004). Similarly, GH may not play an osmoregulatory role in the eel (Sakamoto et al., 1993). Mozambique tilapia can effectively activate branchial ionoregulatory machinery to tolerate SW transfer following hypophysectomy (Breves et al., 2010c), whereas coho salmon (*Oncorhynchus kisutch*) cannot (Björnsson et al., 1987). Even closely related species show differences, as the Nile tilapia that we maintain in our aquarium cannot survive in higher salinity than half strength SW, so we call them stenohaline fish. Nile tilapia increases plasma GH and gill GH receptor transcription after acute SW exposure, which is not observed in the more euryhaline Mozambique tilapia

(Breves et al., 2010b). Species variation linked to phylogeny or life history differences in ion regulatory capacity may have determined whether and to what extent the GH–IGF-I axis is involved in osmoregulation.

3.3. Mineralocorticoids

Cortisol is the major corticosteroid produced in teleost fishes and has a well-established role in salt secretion in euryhaline fishes. The close interaction between cortisol and GH has been detailed above (Section 3.2). In addition to its osmoregulatory function, cortisol plays a role in intermediary metabolism, growth, stress, and immune function (Mommsen et al., 1999). Treatment of a number of euryhaline fishes with cortisol in FW improves their subsequent survival and capacity to maintain low levels of plasma ions after exposure to SW. This effect is due to increases in the size and abundance of gill ionocytes, which have been demonstrated *in vivo* and *in vitro* (McCormick, 2001). Cortisol has also been shown to increase the transcription and abundance of the major transport proteins involved in salt secretion by the gill, especially the SW-type NKAα1b isoform in those species known to contain it, NKCC1 and CFTR (Singer et al., 2003; McCormick et al., 2008; Tipsmark and Madsen, 2009), although it should be noted that these responses are not universal among euryhaline species (Madsen et al., 2007; Tipsmark et al., 2011). Cortisol increases transepithelial resistance and decreases paracellular permeability in the gill, probably working through regulation of specific occludins and claudins (Kelly and Wood, 2002a; Tipsmark et al., 2009; Chasiotis and Kelly, 2011). The effect of cortisol on permeability and tight junction proteins is greater for euryhaline trout than for stenohaline goldfish (Chasiotis and Kelly, 2011). The effect of exogenous cortisol generally requires several days to reach its peak, suggesting that changes in gene expression, cell proliferation, and differentiation are required for its complete action. However, recent studies suggest that some osmoregulatory effects of cortisol may be relatively rapid, less than 1 h (Babitha and Peter, 2010), suggesting a non-genomic action. Rapid, non-genomic actions of corticosteroids in vertebrates are now well established, although the mechanism(s) for these effects, including the presence of a corticosteroid membrane receptor, remain(s) controversial (Losel and Wehling, 2008).

In the intestine, exogenous cortisol stimulates ion and water absorption, thus improving acclimation to high environmental salinity (Hirano and Utida, 1968; Cornell et al., 1994; Veillette et al., 1995). Specific ion transporters that are upregulated by cortisol in the intestine include NKA, NKCC2, and aquaporin 1 and 3 (Seidelin et al., 1999; Martinez et al., 2005; Veillette and Young, 2005; Cutler et al., 2007). Intestinal expression of

claudins 15 and 25b in Atlantic salmon is upregulated by SW exposure but, surprisingly, inhibited by cortisol treatment in FW (Tipsmark et al., 2010b). These authors suggest that contact with imbibed SW may be necessary for full induction of intestinal transport capacity. An increased drinking response after SW transfer has been observed in salmonids treated with cortisol in FW (Fuentes et al., 1996), although whether this is a direct effect on the brain or indirect through other endocrine pathways has not been determined.

Surprisingly little work has been done on the impact of salinity on the kidney. FW exposure resulted in increased NKA activity in the euryhaline mullet (*Chelon labrasus*), but no effect of cortisol was found (Gallis et al., 1979). Cortisol treatment in North African catfish (*Clarias gariepinus*) caused an increased NKA activity in the short term (20 min) but decreased it in the long term (5 days). In an *in vitro* preparation of renal proximal tubules of winter flounder, cortisol significantly increased carbonic anhydrase activity and sulfate secretion (Pelis et al., 2003).

Changes in circulating cortisol in response to increased environmental salinity are reported for many teleost species (Mommsen et al., 1999). The clearance rate of cortisol also increases in SW, suggesting increased utilization by osmoregulatory target tissues. The release of cortisol from the interrenal is primarily controlled by ACTH, although other endocrine factors may also be involved (see Section 2, this chapter). Although there is evidence for salinity activation of pituitary ACTH cells *in vitro*, salinity effects on circulating levels of ACTH have not been detected. ACTH production by isolated pituitary does not appear to be directly responsive to changes in osmolality (Seale et al., 2002). The increase in cortisol during osmotic stress occurs in both stenohaline and euryhaline fishes and may be part of a general stress response. Thus, the regulation of cortisol receptors may represent a critical component of osmoregulation in euryhaline fishes.

The classical signaling action of steroids begins with transport/diffusion into the cell, followed by binding to a cytosolic receptor, which is then translocated into the nucleus. There, the steroid/receptor complex binds to specific genes to increase or decrease their expression. Several studies on cortisol binding in fish tissues have found evidence for only a single class of corticosteroid receptors (CRs) present in high concentrations in gill, gut, and kidney (Mommsen et al., 1999). More recently, two isoforms with differing isoelectric points have been found in gill tissue of the eel, and these are differentially regulated by salinity (Marsigliante et al., 2000a). During exposure to increased salinity, intracellular cortisol and CR levels in the gill shift from the cytosol to the nucleus, indicative of CR binding and translocation (Weisbart et al., 1987). Consistent with direct osmoregulatory

action, high concentrations of CR have been found in gill ionocytes (Uchida et al., 1998).

In the past several years, molecular techniques have demonstrated the presence of two homologues of the mammalian glucocorticoid receptor (GR) and one homologue of the mineralocorticoid receptor (MR) in several teleost species (Bury and Sturm, 2007). The two isoforms of fish "GR-like" genes have different activation affinities for cortisol (Greenwood et al., 2003; Stolte et al., 2006). In addition, at least one cichlid species (*Haplochromis burtoni*) has splice variants of GR2 that have different tissue distributions and cortisol transactivation characteristics (Greenwood et al., 2003). Expression of fish MRs in mammalian cell lines indicated high binding and transactivation efficiency for both aldosterone and 11-deoxycorticosterone (DOC), similar to the binding characteristics of the mammalian MR (Sturm et al., 2005; Stolte et al., 2008). The divergent binding and expression patterns of the GRs and MR in fish suggest different physiological functions, although these have yet to be established.

It has been suggested that DOC, present in the plasma of some teleosts at levels that could activate the fish MR, might be a second mineralocorticoid in fish (Prunet et al., 2006). Injection studies indicate that DOC cannot carry out the SW-adapting functions of cortisol and that cortisol (but not DOC) stimulated both the FW- and SW-dependent NKA isoforms (McCormick et al., 2008). *In vitro* studies indicate that DOC and cortisol have distinct effects on gill transport proteins that vary with salinity, species, and developmental stage (Kiilerich et al., 2011b, c). In these studies cortisol and DOC had similar effective concentrations. Since DOC is present at much lower concentrations than cortisol and does not respond to changes in environmental salinity (Kiilerich et al., 2011a), it seems unlikely that DOC is involved in osmoregulation, at least in rainbow trout. This is supported by studies on an *in vitro* gill preparation of rainbow trout in which cortisol but not DOC increased transepithelial resistance, and both GR and MR antagonists were required to completely block the actions of cortisol (Kelly and Chasiotis, 2011). It should also be noted that there are high mRNA levels of the 11-β hydroxysteroid (including corticoids) metabolizing enzyme genes present in gill tissue, which may have a role in regulating intracellular corticosteroid actions (Nilsen et al., 2008). To date, the weight of evidence indicates that cortisol carries out all or most of the osmoregulatory effects of corticosteroids and acts primarily through a GR, but with some effects occurring through the MR.

Cortisol has been regarded as a SW-acclimating hormone in a large number of teleost species, but there is increasing evidence that cortisol is also involved in ion uptake, indicating that it has dual osmoregulatory functions. Plasma cortisol levels decrease following transfer of salmonids from SW to

FW or after exposure of FW fishes to ion-poor FW (McCormick, 2001). Cortisol treatment of a number of teleost species held in FW increases the surface area of gill ionocytes and the influx of Na^+ and Cl^- (Perry et al., 1992). Survival and plasma ion levels of FW fish that have had their pituitary removed are increased by treatment with ACTH, which can be presumed to be acting through its stimulation of cortisol release from the interrenal (McCormick, 2001). Cortisol is also required to maintain water movement across the gut of FW eels. Cortisol treatment significantly increases the ion regulatory capacity of marine fishes during exposure to low salinity (Mancera et al., 1994) and the ability of acid-resistant fishes to maintain plasma Na^+ levels after exposure to acidic water (Yada and Ito, 1999). Cortisol also upregulates transcription and protein abundance of the FW-dependent NKAα1a isoform in Atlantic salmon gills (Kiilerich et al., 2007a; McCormick et al., 2008). Since cortisol also upregulates the SW-dependent NKAα1b isoform in Atlantic salmon, this is further evidence of a dual osmoregulatory role of cortisol. At least in zebrafish, cortisol also plays an important role in Ca^{2+} balance, and regulates the expression of an epithelial calcium channel (TRPV6) to support active Ca^{2+} uptake by ionocytes (Lin et al., 2011). These studies provide evidence that in at least some teleosts cortisol has a physiological role in acclimation to FW and ion-poor environments. This function of cortisol has not been fully appreciated owing to an emphasis on the role of cortisol in salt secretion.

3.4. Thyroid and Sex Steroid Hormones

There is conflicting evidence regarding the role of thyroid hormones (T_3 and T_4) in osmoregulation, but most studies suggest that they have an indirect role in regulating ion uptake or secretory capacity (McCormick, 2001). Prolonged treatment with T_4 or T_3 accelerates smolt-related increases in gill ionocytes in Atlantic salmon with variable effects on gill NKA activity. Physiological levels of exogenous T_4 and T_3 in Mozambique tilapia result in increased ionocyte size, gill NKA activity, and plasma Na^+ and Cl^- levels, suggesting that thyroid hormones may have a role in ion uptake in this species (Peter et al., 2000). T_3 treatment altered the distribution of gill ionocytes and increased gill NKA activity in FW- and SW-acclimated air-breathing fish *Anabas testudineus* (Peter et al., 2011). Thyroid hormones play at least a supportive role in SW acclimation, and may interact with both the GH–IGF-I and cortisol axes. Inhibition of the thyroid axis with thiourea in killifish caused increased plasma ions in SW but had no effect in FW (Knoeppel et al., 1982). T_4 treatment alone has no effect, but potentiates the action of cortisol on gill NKA activity in Mozambique tilapia (Dange, 1986), and the action of GH on gill NKA activity in Atlantic salmon

(McCormick, 2001). Inhibiting the conversion of T_4 to T_3 interferes with normal and GH-induced SW acclimation in rainbow trout. T_3 treatment increases the number of gill cortisol receptors in trout and salmon (Leloup and Lebel, 1993). Thyroid hormones thus appear to exert their influence on salt secretory mechanisms primarily through an interaction with cortisol and the GH–IGF-I axis.

Sex steroids have been found to have a negative impact on salinity tolerance in salmonids and tilapia (Madsen et al., 1997; Vijayan et al., 2001). Estrogenic compounds have been found to decrease circulating IGF-I levels and gill NKA activity (McCormick et al., 2005). Early developmental exposure can have effects on salinity tolerance a year after exposure, suggesting a possible epigenetic effect (Lerner et al., 2007). The effects of estrogens and androgens in salmonids may be related to their anadromous life history in which departure from the ocean is associated with maturation and elevated androgens and estrogens. In sockeye salmon increases in gill NKAα1a mRNA levels are observed as fish move from the open ocean to coastal waters but before they enter FW, suggesting a preparation for FW entry (Shrimpton et al., 2005). A more complete discussion associated with migration in adult anadromous fishes can be found in Chapter 7 of this volume (Shrimpton, 2013).

4. TARGET TISSUES

Euryhaline fishes maintain water and ion balance in either FW or SW by modulating water and ion exchange at each osmoregulatory organ. As mentioned above, various rapid-acting and slow-acting hormones work in concert to regulate water and ion balance by modulating drinking, intestinal absorption, branchial fluxes, and renal excretion during the whole process of acclimation to changing environmental salinity. This section provides a summary of the hormonal regulation of water and ion trafficking at each osmoregulatory organ.

4.1. Brain Control of Drinking

Drinking is usually suppressed in FW fishes because water enters the body by osmosis and fish are exposed to a constant threat of over-hydration (Takei, 2002; Takei and Balment, 2009). Reflecting the dominance of inhibitory mechanisms in fish, most hormones that regulate drinking are anti-dipsogenic hormones (Ando et al., 2003; Kozaka et al., 2003), except for Ang II, AM2, and IT, as mentioned above. Importantly, the dipsogenic potency of Ang II in teleosts is 1/100th that of mammals

but anti-dipsogenic potency of ANP is 1000-fold more potent in teleosts than in mammals (Takei, 2002). However, drinking is as important in SW fishes as in terrestrial animals because it is the major route for obtaining water that has been passively lost to the environment. In fact, eels with esophageal fistulae die of cellular and extracellular dehydration within 5 days after SW transfer if ingested water is not reintroduced into the stomach (Takei et al., 1998b). Prior to death these eels drank at a rate twice that of intact fish, probably owing to severe hypovolemia and increased plasma Ang II (Takei and Balment, 2009). It is possible that Ang II is involved in the immediate drinking after SW exposure as an ACE inhibitor, SQ14225 (captopril), abolished the immediate drinking induced by SW transfer (Tierney et al., 1995), although the effect of captopril could be due to increased plasma concentration of antidipsogenic bradykinin (Takei and Tsuchida, 2000). It remains to be determined whether the immediate drinking is caused simply by a neural reflex of swallowing triggered by activation of a Cl^- sensor (Hirano, 1974) or by some fast-acting hormones.

The hormone that suppresses excess drinking in SW may be ANP, as its removal from plasma increased drinking in SW eels (Tsukada and Takei, 2006). However, it is possible that ghrelin, another potent antidipsogenic hormone in eels (Kozaka et al., 2003), is secreted from the stomach in response to the increased luminal osmolality or distension by SW drinking and acts on the area postrema to inhibit drinking (Nobata and Takei, 2011). These hormones, together with the nervous signal from the distended stomach, seem to suppress excess drinking and promote SW acclimation (Takei and Hirose, 2002). However, the interaction with other inhibitory hormones in salinity acclimation in euryhaline fishes remains to be determined.

4.2. Intestinal Ion and Water Absorption

As the lumen of the digestive tract is a kind of external environment within the body, water and ions in the lumen become body fluid for the first time after absorption by the intestine. When fish are in ion-poor FW, the intestine actively absorbs ions from food but not water. When they are in SW, ions must also be absorbed in order to obtain water. To achieve this, ingested SW is processed during passage through the digestive tract and water is absorbed in association with monovalent ions when luminal fluid becomes isotonic to the plasma. The initial step of this process is removal of NaCl (desalting) by the esophagus (Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983), but nothing is known about its hormonal regulation. As is the case for regulation of drinking, several inhibitory hormones have been identified for intestinal absorption in SW fishes.

However, no hormone has been elucidated thus far that enhances intestinal absorption of water and ions when administered in isolation *in vitro*, although somatostatin, neuropeptide Y, and catecholamines slightly restore the absorption if it has been suppressed by pretreatment with inhibitory hormones (Ando et al., 2003). Hormones that act alone to facilitate water and ion uptake have not been identified in mammals either, as the absorption is maximally activated to maintain water and ion balance in the desiccative terrestrial environment. The lack of information about the facilitative hormones in fish may be partly because experiments on the intestinal absorption have been conducted in SW fishes in relation to water absorption for SW acclimation. To identify hormones that facilitate ion absorption, FW fish intestine may be a good model as ion absorption is usually suppressed to avoid excess water absorption.

It has been shown that NKCC, NCC, CFTR, and anion exchanger (AE) on the apical side of epithelial cells play major roles in ion absorption by the intestine of SW fishes (Marshall and Grosell, 2006; Edwards and Marshall, 2013, Chapter 1, this volume). There is accumulating evidence showing that active secretion of HCO_3^- into the lumen by the intestinal epithelial cells precipitates $Mg/CaCO_3$ and decreases these ions, thereby decreasing osmolality and further facilitating water absorption (Grosell et al., 2009; Wilson et al., 2009). AE secretes HCO_3^- into the lumen in exchange of Cl^-, which facilitates water absorption. However, if NKCC, NCC, and AE transport Cl^- into the epithelial cells, Cl^- ions in the luminal fluid become deficient, which halts the operation of NKCC and NCC. Therefore, CFTR may supplement Cl^- in the luminal fluid as mentioned above.

It has been shown that ANP inhibits NKCC to decrease NaCl absorption in the intestine of winter flounder (O'Grady et al., 1985). Guanylin stimulates CFTR to secrete Cl^- and HCO_3^- into the lumen of SW-acclimated eels (Yuge and Takei, 2007). Both ANP and guanylin utilize cGMP as an intracellular messenger, although ANP acts from the basolateral side and guanylin from the luminal side. Thus, it is likely that both hormones regulate the same transporters, but only guanylin induces Cl^- secretion as judged by the reversal of short-circuit current. Guanylin may also inhibit NKCC, but ANP does not seem to activate CFTR for Cl^- secretion. It has been suggested that in mammalian intestine guanylin-induced cGMP production not only activates protein kinase G type II but also inhibits phosphodiesterase type III to increase cAMP, and finally activates protein kinase A (Sindić and Schlatter, 2006). This result coincides with the observations that hormones that increase intracellular cAMP such as VIP are inhibitory to NaCl absorption and those that inhibit cAMP production such as somatostatin reverse the inhibitory effect in the teleost intestine.

4.3. Renal Regulation

In terrestrial animals, including mammals, the kidney is the primary organ for body fluid regulation, particularly renal tubules where various hormones are involved in the regulation of ion and water reabsorption. In teleosts, however, urine volume is primarily determined by GFR, and glomerular intermittency (shutting off of some glomeruli) occurs to decrease urine volume by vascular actions. There are some hormones that alter water and NaCl excretion by the kidney, but the changes are mostly ascribed to the glomerular effects. Furthermore, the kidney plays less important roles in volume regulation in SW fishes than in FW fishes. Probably reflecting this difference, ANP exerts only a small antidiuretic effect in SW eels (Takei and Kaiya, 1998) but induces profound diuresis and natriuresis in FW rainbow trout (Duff and Olson, 1986). In addition, Ang II and VT are only mildly antidiuretic via glomerular effects in teleost fishes.

The SW teleost kidney is the site of excretion of divalent ions (Mg^{2+}, Ca^{2+}, and SO_4^{2-}), and these ions are secreted into the proximal tubular lumen via various transporters (Beyenbach, 1995; Renfro, 1999; Edwards and Marshall, 2013, Chapter 1, this volume). Mg^{2+} and SO_4^{2-} concentrations in SW are 50- and 30-fold higher, respectively, than those in plasma. Passive influx is generally negligible but unavoidable influx is balanced by the active secretion in the kidney. Recently, the transporters involved in SO_4^{2-} uptake in FW fishes and SO_4^{2-} excretion in SW fishes have been identified and localized in the kidney: FW eel (Nakada et al., 2005), SW euryhaline pufferfish (*Takifugu obscures*) (Kato et al., 2009), and SW eel (Watanabe and Takei, 2011b). Cortisol has been shown to increase SO_4^{2-} excretion in an *in vitro* preparation of the renal proximal tubule of winter flounder (Pelis et al., 2003). It is likely that renal regulation of divalent ions is of primary importance for acclimation in both FW and SW (euryhalinity), but little else is known about the hormonal regulation of divalent ions in the kidney of fish.

4.4. Branchial Regulation

It is obvious that the gills are a critical osmoregulatory organ in fishes as this organ is directly exposed to environmental water with monolayer respiratory epithelia and thus serves as a window for communication with the environment. Several slow-acting hormones (GH/IGF-I and cortisol) have been implicated in the transformation of ionocytes between an absorptive FW type and an excretory SW type, but the information about the role of rapid-acting hormones in acute regulation of ion fluxes is still

limited. It has been reported that VT and IT inhibited cAMP production in the gill cell membranes from rainbow trout (Guibbolini and Lahlou, 1987), and ANP and UII increase Cl^- secretion in the killifish opercular membrane, probably via ionocytes (Marshall and Bern, 1981; Scheide and Zadunaisky, 1988). However, the effects are controversial in other species and remain to be established. As several rapid-acting hormones stimulate GH and/or cortisol secretion, they may have indirect actions on the gills to promote differentiation of ionocytes between FW and SW type, as mentioned above.

As noted in detail above (Sections 3.2 and 3.3), cortisol and the GH/IGF-I axis interact to promote the differentiation of salt-secreting ionocytes in the gill, including the upregulation of the three major transport proteins involved: NKA, NKCC1, and CFTR. This interaction is determined at least in part by the effect of GH on cortisol receptors and responsiveness of the interrenal to ACTH. Prolactin and cortisol are each involved in ion uptake, and act to promote the differentiation of FW ionocytes, and at least some of the transporters involved, such as the apical NCC. There is increasing evidence of an interaction between cortisol and prolactin in regulating gill ion uptake, although the universality and mechanism of this interaction remain to be established.

5. DEVELOPMENTAL (ONTOGENIC) ASPECTS

Diadromous fishes are amphihaline, but the degree of euryhalinity or adaptability to different salinity environments changes during development. During the parr–smolt transformation of salmonids or the "silvering" stage of eels, there are large increases in SW tolerance in association with downstream migration (McCormick, 2013; Zydlewski and Wilkie, 2013, Chapters 5 and 6, this volume). Salmonids and eels have been widely used for the study of osmoregulation and euryhalinity, and it is often found that the endocrine regulation of osmoregulation differs between the species. This may derive from the fact that salmonids probably have their evolutionary origins in FW and are anadromous, whereas eels have an SW origin and are catadromous. Salmonids have been widely used as a model for developmental changes in hypoosmoregulatory ability, and differences in the timing of smolt development provide useful comparative approaches. Smolting appears to be a "pan-hyperendocrine" event, and details of the hormones involved can be found in Chapter 5 of this volume (McCormick, 2013). Eels can be used for the developmental aspect of hyperosmoregulatory ability,

but research on the complete aquaculture of eels from eggs has yet to be completed (Tanaka et al., 2003).

Medaka (genus *Oryzias*) are mostly non-migratory fishes of FW origin, but there are a variety of species that exhibit distinct adaptability to SW (Inoue and Takei, 2002). *Oryzias mormoratus* is a species endemic to a highland lake of Sulawesi Island, Indonesia, and fertilized eggs and adult fish cannot survive in more than half-strength SW. *Oryzias javanicus* is distributed around the coastal area of the Indonesian Islands and Malay Peninsula, and even fertilized eggs develop normally in SW. *Oryzias latipes* lives in the rice fields of Japan and is intermediate in terms of salinity tolerance. It cannot survive direct transfer from FW to SW but can live in double-strength SW if acclimated gradually. The eggs can be fertilized in SW and develop normally until hatching (Inoue and Takei, 2002). It has been shown that the yolk-sac membrane of euryhaline Mozambique tilapia embryos has many ionocytes, which are transformed from FW-type to complex SW-type after transfer of embryos to SW (Hiroi et al., 1999). As embryos cannot gain water by drinking and intestinal absorption, they seem to limit water efflux from the body surfaces and produce sufficient oxidative water by active yolk metabolism to balance the obligatory osmotic water loss.

It is interesting to examine how medaka embryos cope with dehydration during early developmental stages and which hormones are involved in the development of adaptability. For this purpose, a knockdown technology using antisense oligonucleotide (gripNA) was applied to BNP, as medaka have only BNP as cardiac NPs and therefore ANP and VNP cannot compensate for the BNP function in this species (see Section 2.2, this chapter). The BNP gene starts to express in the primordial ventricular tissue at 48 h postfertilization (hpf) and its receptor (OLGC7) gene transcripts appear even earlier, at 10 hpf (Miyanishi et al., 2013a). CNP3, which is an ancestral molecule of cardiac NPs but usually synthesized in the extracardiac tissue, is expressed in the developing atrium at 35 hpf and its receptor (OLGC2) at 10 hpf. After knockdown of the BNP gene, no change was observed in the developing embryos, but a double knockdown of the BNP and OLGC7 genes severely impaired the normal ventricular development (Miyanishi et al., 2013a). By contrast, knockdown of the CNP3 gene alone caused abnormal atrial development. These results clearly show that BNP and CNP3 are important not only for osmoregulation but also for normal development of cardiac tissues, and that CNP3 compensates for the function of ANP for atrial development in medaka embryos. Furthermore, knockdown of BNP or CNP3 gene increased body fluid osmolality of embryos kept in SW compared to controls (Miyanishi et al., 2013b). The increase was found to be due to impaired blood flow to the

yolk-sac membrane, which leads to malfunction of NaCl secretion by ionocytes and reduced yolk metabolism for metabolic water production. However, expression of the major transporter genes involved in NaCl secretion by ionocytes (NKA, NKCC1a, and CFTR) and expression of the key metabolic enzymes for energy metabolism were not suppressed by the knockdown. The increase in body fluid osmolality was further exaggerated in the later stage of development after CNP3 knockdown, which was due to the failure to suppress the expression of aquaporin (AQP3, 4, and 9) genes by the loss of CNP3 (Miyanishi et al., 2013b) as observed in the ANP/NPR-A knockout mouse (Kishimoto et al., 2011).

6. EVOLUTIONARY (PHYLOGENETIC) ASPECTS

6.1. Cyclostomes (Lampreys)

Early vertebrates are thought to have evolved in the SW environment near the coast, but basal fishes might have once entered the FW environment and then re-entered the sea (Carroll, 1988; Schultz and McCormick, 2013, Chapter 10, this volume). However, one of the two most basal extant vertebrates, hagfishes, does not appear to have experienced FW during their evolutionary history, as judged by their simple kidney structure. Therefore, they live in the deep sea and are strictly stenohaline with a plasma ion composition almost identical to SW except for divalent ions. By contrast, lampreys are FW or anadromous species that have an osmoregulatory strategy similar to teleosts. Such large differences in basic physiology support the idea that the two cyclostome species diverged long ago in vertebrate phylogeny. Nonetheless, the mechanisms of euryhalinity and their hormonal regulation in lampreys may provide us with a prototype of the earliest aspects of osmoregulatory control in vertebrates.

Embryonic anadromous lamprey hatch in rivers and are known as ammocoetes in their FW stage; they cannot survive in water with salinity higher than their body fluids (Reis-Santos et al., 2008). However, metamorphosing juveniles (transformers) obtain excellent euryhalinity and can maintain low plasma osmolality even in SW. Transformers have much more abundant (approximately 10-fold) NKA activity in the gills than do ammocoetes, which may in part explain their SW adaptability, while H^+-ATPase and carbonic anhydrase may be responsible for ion uptake in FW (Reis-Santos et al., 2008). Several rapid-acting, peptide hormones such as VT (Suzuki et al., 1995), Ang II (Wong and Takei, 2011), NPs (Kawakoshi et al., 2006), and AMs (Wong and Takei, 2009) have been identified in lampreys, but their osmoregulatory functions have not been examined yet.

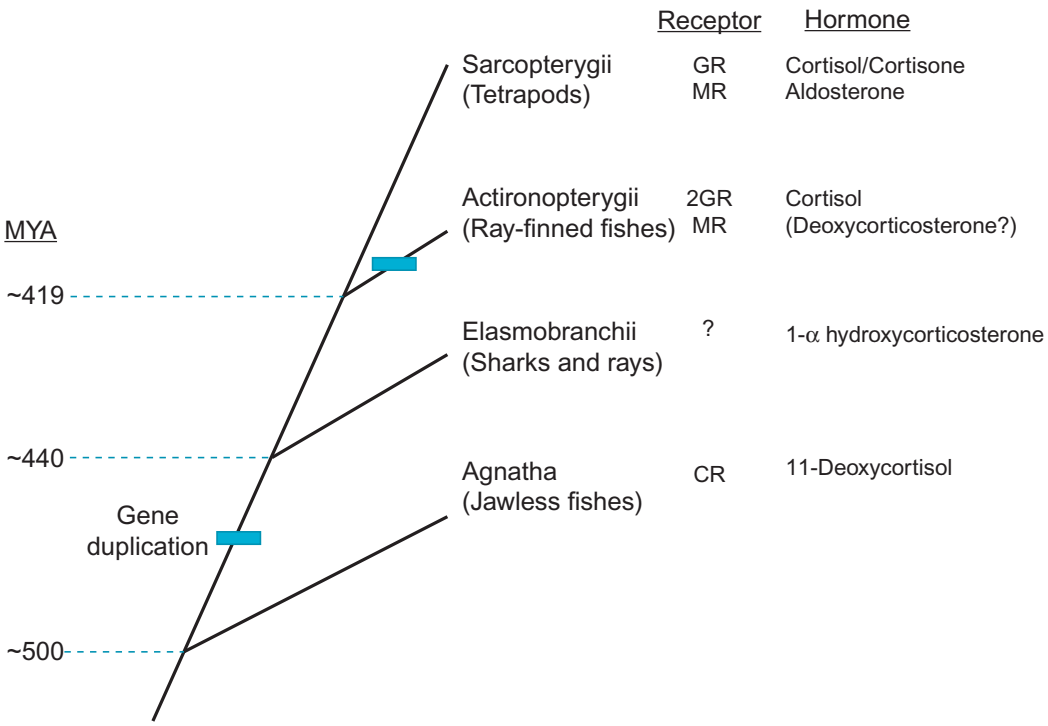

Fig. 3.3. Phylogenetic analysis of corticosteroids and their receptors in vertebrates (modified from Close et al., 2010). Blue boxes indicate hypothesized whole genome duplication events. MYA, million years ago; CR, corticosteroid receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

It has recently been determined that 11-deoxycortisol is the major corticosteroid of lamprey and has both osmoregulatory and metabolic functions (Close et al., 2010). Lampreys appear to have a single CR, consistent with their origination prior to the gene duplication event of other vertebrates (Fig. 3.3). Heterologous expression studies suggested that this receptor can respond to a variety of corticosteroids (Bridgham et al., 2006), but binding and physiological studies indicate that the receptor is highly specific for 11-deoxycortisol (Close et al., 2010). Unlike other metamorphic events, thyroid hormones are inhibitory to the metamorphosis of ammocoetes up to the parasitic transformer stage (Youson, 2003). It will therefore be of interest to examine the relationship between 11-deoxycortisol and thyroid hormone during events when salinity tolerance develops.

Lampreys apparently have only one protein hormone of the GH-PRL-SL family (Kawauchi et al., 2010). This has been suggested to be GH based on its sequence similarity with other vertebrate GHs and its ability to induce IGF-I production. To date, there is no published information on the possible role of this hormone or its receptor in osmoregulation.

6.2. Elasmobranchs

Although most elasmobranchs are stenohaline marine species, a number of euryhaline species migrate between FW and SW (Ortega et al., 2009; Evans et al., 2010) or even live wholly in FW (Ballantyne and Fraser, 2013, Chapter 4, this volume). Elasmobranchs and holocephalans display an osmoregulatory strategy in which they accumulate urea and trimethylamine oxide in their body fluids to counter osmotic water loss in SW. Euryhaline elasmobranchs retain NaCl and urea in plasma at high concentrations after acclimation to FW (Piermarini and Evans, 1998; Pillans and Franklin, 2004), which differs from stenohaline SW species such as *Scyliorhynus canicula*, in which transfer to diluted SW decreases plasma NaCl and urea concentrations linearly (Wells et al., 2002). Stenohaline FW rays in the Amazon River (*Potamotrygon* spp.) have lost plasma urea and maintain plasma NaCl at much lower concentration than that of euryhaline species in FW (Ballantyne and Robinson, 2010). Therefore, it appears that the ability to maintain high plasma NaCl and urea concentrations in low-salinity media is the key to euryhalinity in elasmobranchs.

It has been shown that maintenance of plasma NaCl at a concentration lower than SW is achieved by rectal gland secretion (Piermarini and Evans, 2000) and that high plasma urea concentration is maintained by active synthesis of urea in the liver and skeletal muscle (Kajimura et al., 2006) and facilitated reabsorption of urea by the collecting tubule of the kidney (Hyodo et al., 2004a; Yamaguchi et al., 2009). Elasmobranch gills also play important roles in body fluid regulation as a site of ion uptake in FW and as a barrier against the loss of ammonia and urea (Hazon et al., 2003). Various transporters have been identified in these osmoregulatory organs (Silva et al., 1997; Piermarini et al., 2002; Hyodo et al., 2004a). Detailed accounts of transporters involved in elasmobranch osmoregulation can be found in Chapter 4 of this volume (Ballantyne and Fraser, 2013), and thus only hormonal regulation will be described in detail here.

There are relatively few studies on the hormonal control of elasmobranch osmoregulation, and thus only few firm conclusions are possible (Good and Hazon, 2009). Plasma Ang II concentration increased transiently 12 h after transfer of bull shark from FW to 75% SW (Anderson et al., 2006). Ang II has been shown to participate in various aspects of osmoregulation in elasmobranchs (Hazon et al., 1999). Ang II is dipsogenic in *Triakis scyllium*, and pharmacological activation or inhibition of the RAS increased or decreased drinking rate, respectively, in *S. canicula* (Anderson et al., 2001). Dense Ang II binding sites have been identified in the *Triakis* gill (Tierney et al., 1997). More recently, an Ang II receptor (AT1-like) has been identified in the euryhaline Atlantic stingray (*Dasyatis sabina*), the gene of

which is most abundant in the interrenal tissue, followed by the kidney, gills, and rectal gland (Evans et al., 2010). Ang II infused into the *in situ* perfused trunk preparation of *S. canicula* caused glomerular antidiuresis with a parallel decrease in perfusate flow, indicating constriction of preglomerular afferent arteries (Wells et al., 2006). Ang II had no effect on rectal gland secretion in *S. canicula* (Anderson et al., 2002).

CNP3 is the sole member of the NP family in elasmobranchs and its gene is expressed in both the brain and heart (Kawakoshi et al., 2001). Plasma CNP3 concentration does not change for 4 days after transfer of bull shark from FW to 75% SW but it is significantly higher in SW-acclimated fishes (Anderson et al., 2005, 2006). The CNP3 receptor (NPR-B) gene is expressed most abundantly in the rectal gland, followed by the kidney and interrenal gland of Atlantic stingray (Evans et al., 2010). CNP3 is a powerful stimulator for rectal gland secretion in *S. acanthias* (Solomon et al., 1992) and *S. canicula* (Anderson et al., 2002). In contrast to Ang II, CNP3 induced glomerular diuresis in the perfused trunk preparation of *S. canicula* (Wells et al., 2006).

Hypothalamic VT gene expression is enhanced in *T. scyllium* after transfer of fish from SW to 130% SW, with a parallel increase in plasma VT concentration (Hyodo et al., 2004b). Plasma VT concentration also increased 3 days after transfer of bull shark from FW to 75% SW (Anderson et al., 2006). VT infused into the *in situ* perfused trunk preparation caused glomerular antidiuresis with a parallel decrease in perfusate flow, indicating constriction of preglomerular afferent arteriole as observed with Ang II (Wells et al., 2002). In addition, VIP has been reported to act as a potent secretagogue in the rectal gland of *S. acanthias* (Stoff et al., 1979). A stimulatory gut peptide for rectal gland secretion was first reported in *S. canicula* and named rectin (Shuttleworth and Thorndyke, 1984). Anderson et al. (1995) purified and sequenced the possible rectin from the intestine of *S. canicula* and showed this factor to be the previously identified intestinal tachylinin, scyliorhinin II. The possible role of these gut peptides in elasmobranch osmoregulation has been reviewed recently (Good and Hazon, 2009; Takei and Loretz, 2011).

A single, unique corticosteroid, 1α-hydroxycorticosterone, appears to be the major corticosteroid of elasmobranchs (Turscott and Idler, 1968). Receptors to this hormone are present in the gills and rectal gland (Hazon et al., 2003). Surgical removal of the interrenal in the ray (*Raja ocellata*) resulted in reduced ion secretion by the rectal gland which was restored by 1α-hydroxycorticosterone (Holt and Idler, 1975), suggesting an osmoregulatory role for this corticosteroid. To date, there is no published information on how circulating levels of this hormone change in response to external salinity, or its mechanism(s) of action in osmoregulatory tissues. Similarly,

there is little information on how pituitary hormones may be involved in osmoregulation in elasmobranchs. Hypophysectomy results in reduced branchial water permeability and urine flow; branchial permeability is restored by both ACTH and prolactin treatment, whereas only ACTH restores urine flow (Payan and Maetz, 1971). Much remains to be investigated for hormonal control of the unique osmoregulation in cartilaginous fishes.

7. CONCLUSIONS AND PERSPECTIVES

Fish have the greatest species diversity among vertebrates and have acclimated to almost all aquatic habitats. Teleosts are a favored food item and often cultured in a wide variety of salinities, so it is not surprising that most of the research on the hormonal control of osmoregulation has been performed in euryhaline teleost species. However, our understanding of euryhalinity will be broadened by an increased understanding of the physiological mechanisms of ionoregulation in stenohaline FW and marine species for comparison with those of euryhaline species. In addition, migratory lampreys have a similar life history and osmoregulatory strategy to anadromous salmon, and comparison of the major hormones that allow them to adapt to both FW and SW should give us important clues to the evolutionary changes in hormone function. Similarly, increased understanding of the hormonal control of osmoregulation in other basal fishes such as holocephalans and chondrosteans will help to fill the large knowledge gaps in our understanding of the evolution of euryhalinity.

In the study of euryhalinity, the regulation of monovalent ions (Na^+ and Cl^-) has been the major focus as these ions are the dominant extracellular ions that change quickly after transfer between FW and SW. However, the concentration gradient of divalent ions, Mg^{2+} and SO_4^{2-}, between plasma and the external environment is even greater than that of Na^+ and Cl^-. Therefore, amphihaline fishes must alter the way in which divalent ions are regulated even more drastically when they migrate between FW and SW (Watanabe and Takei, 2011a). In the teleost kidney, cortisol has been shown to stimulate SO_4^{2-} secretion by stimulation of carbonic anhydrase (Pelis et al., 2003), and SO_4^{2-} transporters involved in reabsorption in FW and excretion in SW have been identified (Nakada et al., 2005; Kato et al., 2009; Watanabe and Takei, 2011b). Determining the endocrine pathways that regulate renal transporters is a critical next step of investigation.

There are closely related species within the same genus whose genome and phenotype are highly similar but that have very different degrees of

euryhalinity. These include the genus *Oreochromis* (the stenohaline Nile tilapia and the euryhaline Mozambique tilapia), *Oryzias* (the stenohaline *marmoratus* medaka and the euryhaline *javanicus* medaka), anadromous salmonids that migrate at different times after hatching, and *Fundulus* (stenohaline marine and FW killifish and euryhaline mummichog; see Griffith, 1974). Migratory and landlocked salmonids of the same species also exist. Genome projects in some of these species are ongoing, and the recent development of next generation sequencing will accelerate the completion of the projects. Comparison of hormone genes and their promoter regions between the euryhaline and stenohaline species (such as occurrence of mutation in the osmoregulatory hormone genes) will enable us to gain new insights into the key hormones for euryhalinity. Furthermore, transcriptome analysis using next generation sequencers to compare the gene expression profiles after salinity challenges between euryhaline and stenohaline species will elucidate the major genes responsible for rapid and slow responses including hormone genes (Whitehead et al., 2011). Proteomics and other approaches that allow the accurate measurement of small quantities of hormone transcripts should also provide a needed link between genomics and the more physiologically relevant hormone and protein production. There have been many recent advances in imaging techniques allowing analysis of ion movements, hormone secretion, and endocrine-driven differentiation of cell types within osmoregulatory tissues. We expect that the application of new technologies combined with classic endocrine approaches to fish studies will open a new field of research for understanding the hormonal control of euryhalinity.

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