Osmoregulation in Estuarine and Intertidal Fishes

Abstract

Intertidal and estuarine fish stand out among euryhaline fish because of their physiological plasticity in response to frequent salinity changes and other environmental challenges, including polar ice and tropical heat. The Northern killifish *Fundulus heteroclitus*, a scientific model species, and the tropical Nile tilapia *Oreochromis niloticus* that is widely grown in aquaculture, are featured as examples. Estuarine fish combine low water permeability of skin and gill epithelia with efficient NaCl secretion to live in seawater and hypersaline conditions but have variable abilities to absorb NaCl from dilute environments, with some species requiring dietary salt intake for survival in freshwater. Changing salinity produces temporary shifts in plasma osmolality, and osmosensing ionocytes respond by increasing NaCl secretion if shrunken and shutting off secretion if swollen osmotically. Aerial stranding and semi-terrestrial living in climbing species stresses respiratory, osmoregulatory and acid-base systems, involving large coping abilities. In some cases, estuarine fish will go through a tolerance phase of one to two tidal cycles before launching true acclimating mechanisms, so the response to cycling salinities is slight. This curious delay in acclimation responses calls for more

investigations through genomic and proteomics, methods that are becoming more widespread, as more genomes of estuarine species are completed.

1. Introduction

The focus of this review is on estuarine and tide pool resident teleost fish species and includes some well-studied model species such animals as mummichog (*Fundulus heteroclitus*), Nile tilapia (*Oreochromis niloticus*), mudskippers (*e.g. Periopthalmodon modestus*), some euryhaline flounders (*e.g.* starry flounder, *Platichthys stellatus*), stickleback (*e.g.* threespine stickleback, *Gasterosteus aculeatus*), silversides (Atlantic silverside, *Menidia menidia*), sculpins (*e.g.* the tide pool sculpin *Oligocottus maculosus* and coastal prickly sculpin, *Cottus asper*), intertidal blennies (*e.g. Blennius pholis*) and gobies (e.g. longjaw mudsucker, *Gillichthys mirabilis*). These species stand out because of their physiological plasticity in response to salinity changes and other environmental challenges and the wide use of a few of these species as models for osmoregulatory ability. We exclude species that transiently pass through estuaries on migrations (eels, salmonids, bass, herring, alewife) or that use estuaries as nursery habitat, covered by chapters 5 and 6, this volume (Takei and McCormick, 2013;Zydlewski and Wilkie, 2013). The environmental range includes estuaries, the intertidal zone and tide pools, where there are many observed and often large variations in salinity. In estuaries, haloclines and salinity gradients can develop, such as from rain and runoff that reduce salinity. In the intertidal zone some fish allow themselves to be stranded, a habit that can produce physiological challenges, including desiccation, gas exchange and nitrogenous waste excretion. In tide pools, rain can reduce salinity, while evaporation can produce hypersaline conditions. The topic centers on the special adaptations that have evolved in these animals to cope with their highly variable environment. Some of these euryhaline estuarine forms (*e.g.* brackish water mummichog) are essentially marine fish that tolerate freshwater (FW) well (Whitehead et al., 2011), while others, such as Nile tilapia, tend to be FW-like forms that can, if challenged, develop salt secretory mechanisms (Guner et al., 2005;Inokuchi et al., 2009).

2. Intertidal habitats: Estuaries and Tide pools

2.1 Physical characteristics

Estuaries are characterized as brackish water intermediate zones between riverine habitat and the oceans. The size of an estuary depends on the shallowness of the slope and the size of the river(s) feeding the upstream end of the estuary. Thus the largest estuaries are on the order of a million hectares and small streams may have less than a hectare. In most cases, salinity of the surface water is less than at the bottom and, because of the general roughly triangular shape with the narrow end pointing upstream, the incursion of dense cold seawater (SW) underneath the brackish upper layers, these are called wedge estuaries (Figure 1)(Acha et al., 2008). The halocline and thermocline can be stable over time and very sharp, such that salinity can rise from FW at the surface to essentially full strength SW in a depth of 1-2 m. Wedge estuaries, where typically a wedge of cold (in temperate zones), high density SW occupies the deeper parts of the estuary, are made more stable during neap tides where mixing and shear stresses are smaller, but during spring tides the incursion of the salt wedge progresses higher into the estuary and is more mixed, the result of the higher mixing energy of the larger tidal flux (Simons et al., 2010). In models of moderate-sized estuaries with large tidal flux, the salt wedge at the bottom

of the estuary strengthens during neap tides and stratification decays during more energetic spring tides (Wang et al., 2011). Strong wind events also can reduce stratification and produce well-mixed conditions (Acha et al., 2008). Generally, stratification of estuaries enhances the intensity of haloclines such that fish moving vertically in an estuary can encounter large changes in salinity over short distances.

Many estuaries have barrier dunes and islands that separate the main body of estuary from the ocean; in these cases the stratification and salt wedges are restricted to the occasional river-like openings to the ocean and the majority of the estuary is a weakly brackish lagoon. In these cases, fish moving into or out of the estuary would experience a change from brackish to full strength SW, sometimes accompanied by temperature shock. Thus, in most estuaries, resident fish species will experience salinity change with tidal flux, when they move through the estuary and on leaving and entering the estuary.

Tide pools are small bodies of SW captured as the tide ebbs that are exposed to warming from the sun, exposure to the cold in winter but less wave action. Biological communities are highly variable and differ with wave exposure (open coast versus inner bays), vertical position in the intertidal zone and tidal amplitude (Jordaan et al., 2011). The pools that include fish are typically isolated from the ocean for 6-10 h until the tide returns, as opposed to pools in the spray zone that can be isolated for weeks, but do not contain fish. Salinity in coastal marine tide pools thus begins as full strength SW and if the pool is isolated from the ocean for several days there can be evaporative concentration often resulting in hypersaline conditions, warming and hypoxia. There are reports of salinities up to 76 ‰ (2.4 x SW) in salt marsh pools where the gulf killifish (*Fundulus grandis*) is found (Genz and Grosell, 2011). Alternatively, isolated tide pools can become diluted from rain and run off and become brackish or freshwater. These tide pools are suddenly cooled and the salinity restored to full strength SW when the tide returns. Estuarine tide pools are typically brackish. Fish that preferentially inhabit tide pools are generally small, camouflaged and are benthic or suprabenthic in habit. Pelagic fish species are occasionally trapped in tide pools and thus technically are part of the community (Jordaan et al., 2011).

2.2 Climatic extremes

2.2.1 Polar

Estuaries in the Baltic, Gulf of St Lawrence and points northward form thick pack ice covers that minimize light and oxygen availability, while the haloclines remain intact. In winter, marine fishes such as flounder seek estuaries because the estuarine waters are warmer (1^{o}C) than the ocean (-1.5 ^{o}C) (Hanson and Courtenay, 1996). Different species react differently to the cold, some maintaining osmoregulation and activity, others becoming inactive. Intertidal zones in locations with seasonal ice cover endure severe cold and scouring of the shoreline, making year-round occupation by fish impossible. Only the lower littoral zone has significant vegetation and few animal species. In these zones, intertidal fishes generally do not migrate large distances, but rather seek coastal subtidal refuges for overwintering. One strategy is for the fish to occupy the deeper parts of estuaries in protected water. Alternatively, estuarine fish can migrate moderately upstream where warmer freshwater streams run all year. The former strategy means that the animals must survive in cold, full strength SW. The second strategy involves survival in cold FW. In either case, survival requires strong eurythermic and euryhaline capabilities.

2.2.2 Tropical

Tropical estuaries can stratify and produce haloclines and, unlike their temperate counterparts, can have inverted haloclines that trap warm saline water on the bottom (Stith et al., 2011). In tropical estuaries, mangrove swamps have extreme heat and low oxygen levels, conditions that force many species to either evolve accessory breathing specializations and/or become semiterrestrial. There appear to be only slight osmoregulatory consequences of air breathing in teleosts and many species revert to surface breathing in hypoxic conditions. Terrestrial sorties can be for breeding, foraging or predator avoidance and the fish still retain osmoregulatory abilities when they return to the water. Of the mangrove fish species, mangrove killifish and mudskippers are particularly well studied. These aerial species rely on cutaneous gas exchange and maintain acid-base balance by a suite of adaptations, including (1) active excretion of NH_4^+; (2) lowering of environmental pH; (3) low NH_3 permeability of epithelial surfaces; and, in a minority of species (4) volatilization of NH_3 (Chew et al., 2006;Ip et al., 2004) and all are subject to evaporative water loss during terrestrial sorties (see below).

3. Osmoregulatory Strategies

3.1 Thermodynamics

An analysis of the ion and osmotic gradients at different salinities compared to blood plasma and to cytosol of ion transporting cells of the gill epithelium illustrates the gradients against which estuarine fish must ion- and osmoregulate. Several authors have presented this in different ways (Evans, 1984;Potts, 1984) but always with ion concentrations expressed on a linear scale. Here we consider the ion gradients across the gill as fold gradients favoring inward (+) or outward (-) ion movement and as their Nernst equilibrium potentials (as mV), imagining that the membrane is conductive to one ion (Figure 2a,b). The Nernst equilibrium potential for a monovalent ion is: $\Delta\psi = (RT/F)\ ln[C_o/C_i]$, where R is 8.3143 Coul. V/^{o}K mol, T is ^{o}K, *F* is 96,494 Coul./mol. Equiv. and C is the ion concentration on the inside, i, and outside, o, of the membrane, respectively. The log scale helps expand the dilute end of the environmental concentration, thus allowing visual distinction between normal FW, at approx 1 mM NaCl, and ion-poor environments. This distinction becomes important because few estuarine fish can survive in ion-poor environments. In the top of Figure 2 is a graphical interpretation of blood plasma ion concentrations versus environmental concentrations in a log-linear plot that keeps the equilibrium potentials linear, while the "isoionic" line, now steeply curved, delineates the border above which the fish hyperosmoregulates and below which the animal hypoosmoregulates.

In the bottom half of the figure (Figure 2b) are expressed the transmembrane concentrations (approximated, because many have not been measured) and the local apical membrane concentration gradients and equivalent electrical potentials in mV. We see that in FW the gradients can exceed 100-fold and the equivalent voltages rise above 120 mV, well above the normal physiological range of transmembrane potentials. Na^+ transmembrane gradient is smaller than the equilibrium potential in FW in the range of 1.0-10.0 mM Na^+, implying that a simple apical membrane Na^+ conductance by itself could not produce Na^+ influx (uptake). In brackish water above 10 mM NaCl, estuarine fish use a neutral ion uptake mechanism, NCC or ion exchange to uptake Na^+ and Cl^- (see section 5) and review by (Evans et al., 2005;Evans, 2011). In ion-poor conditions, the higher affinity NaCl uptake mechanisms involving V type H^+-ATPase are necessary, yet

most estuarine teleosts do not utilize these pathways (see section 5), thus limiting their access to some FW environments. On the SW side, at the apical membrane the presumed intracellular ion activities are lower than the environment, thus producing positively-directed gradients of 11 to 31-fold and E_{Na} of +87 mV (far from the estimated transmembrane voltage of -60 to -80 mV) but well within the normal range for E_{Cl}, thus showing that Cl^- can be secreted easily into SW simply via an apical membrane conductance, limited in marine teleosts to the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel (see also section 5). The apical membrane voltage has not been accurately measured (see below), but in chloride secreting systems that are more amenable to intracellular impalement, such as corneal epithelium of rabbit, the intracellular potential at the apical membrane is -50.9 $\pm$ 1.0 mV n = 121, the voltage drop through the tissue is an additional -6.0 mV (for a total of -56.9 mV) and the basolateral voltage is -78.2 $\pm$ 0.6, with the transepithelial voltage being the difference, +21.4 $\pm$ 0.8 mV (Marshall and Klyce, 1983). Thus there is sufficient TEP to drive Na^+ secretion paracellularly (see below) as well as transcellular Cl^- secretion across the apical membrane. In airway epithelium, NKCC operation on the basolateral side causes intracellular Cl- to accumulate to 39 $\pm$ 3 mM, well above the Nernst equilibrium potential of 17 $\pm$ 2 mM; the apical membrane voltage is -60 $\pm$ 2 and decreases to -46 $\pm$ 2 mV in resting versus secreting airway epithelial monolayers, reviewed by (Welsh, 1986).

Na^+ efflux follows a paracellular pathway and requires a transjunctional E_{Na} of +32 mV, less than the measured transepithelial potential seen in some marine systems (+37 to +40 mV, (Guggino, 1980;Pequeux et al., 1988). Thus there is sufficient electrochemical driving force to secrete Cl^- transcellularly and Na^+ paracellularly into SW. The secretion of Cl^- across the apical membrane into hypersaline solutions has not been examined energetically before, to my knowledge, and it does present some mechanistic challenges. Either intracellular Cl^- needs to rise and/or the intracellular potential has to deepen to larger negative values. The former would be produced by more NKCC activity and the latter would be produced by higher basolateral K^+ conductance and either requires higher NKA activity. In hypersaline conditions the same pattern could be sustained simply if basolateral K^+ conductance were increased with a concomitant increase in NKA expression and activity. If K^+ conductance were to hyperpolarize by 50%, the transmembrane potential would increase to $^-$85 to $^-$90 mV and the maximum salinity into which the animal could secrete Cl^- would be almost 2M Cl^-, a level readily obtained by teleosts and invertebrates such as brine shrimp (Griffith, 1974).

The log-linear presentation helps perceive the ion gradients in dilute media, such that transposed data for a group of related intertidal blenniid species shows clearly the divergence of osmoregulatory ability in dilute media (Figure 3). Mummichogs, the most hardy of killifish, can sustain osmoregulation in salinity equivalent to 1966 mM Cl^- (Griffith, 1974), so an extreme voltage gradient would be necessary to secrete Cl^- into that medium. Importantly, the transmembrane voltages at the apical and basolateral membranes of ionocytes have never been measured accurately. A serious attempt to impale ionocytes obtained rather small potentials of only -18 mV (Zadunaisky et al., 1988) but it is likely that the complex tubular system of ionocytes would be damaged by microelectrode impalement thus degrading the measured potential. Perhaps noninvasive methods could obtain the necessary values for these potentials. The transepithelial (transbody) potentials, however, are easily obtained and are often measured.

3.2 Electrical potentials

Transbody electrical potentials can be measured easily *in vivo* and demonstrate ion selectivity of the gill epithelium, the organ with the largest surface area and greatest overall permeability. In SW, estuarine teleosts, including European flounder, (*Platichthys flesus*), blenny (*Blennius pholis*), longjaw mudsucker, mozambique tilapia (*Oreochromis mossambicus*) and mummichog generally have inside positive potentials in the range of +10 to +35 mV (Potts, 1984;Potts et al., 1991) and their approximation to the E_{Na} strongly suggests that the Na^+ conductance of the gill is very large relative to anions such as Cl^-. In European flounder, the TEP comprises a large diffusional component and a smaller electrogenic active component (Potts et al., 1991). In a study of Mozambique tilapia the measured transbody potential actually exceeded the E_{Na}, demonstrating that Na^+ may be secreted passively by a favorable electrochemical gradient (Dharmamba et al., 1975). In larval mummichog, animals where the ionocytes are in the yolk sac membrane and the gill has not yet developed, the measured transbody potential was consistently higher than the E_{Na}, +37 to +40 mV (Guggino, 1980). In isolated mummichog opercular membranes, without the shunting effect of the gill lamellae (Wood and Grosell, 2008;Wood and Grosell, 2009), and in asymmetrical conditions with SW on the mucosal side, the transepithelial potential is again higher than E_{Na} (+28.8 mV), averaging +36.8 mV (Pequeux et al., 1988), illustrating that there is sufficient driving force for Na^+ to be secreted passively down its electrochemical gradient, if it traverses a paracellular pathway. Although it is technically difficult, it would be ideal to measure the local electrical potential adjacent to the apical crypts of ionocytes to demonstrate that Na^+ exit form SW fish is via passive diffusion down the Na^+ electrochemical gradient. The calculated E_{Cl} is universally negative in polarity (Figure 2) and a few species of fully marine teleosts have measured transbody potentials with this polarity, reviewed previously (Evans et al., 1999;Potts, 1984). The negative inside potentials in seahorse (*Hippocampus erectus*) and toadfish (*Opsanus beta*) still call for further investigation as to how these species secrete NaCl into SW. The transbody potential does demonstrate indirectly that there is a predominant cation (Na^+) permeability in marine fish and that there is much less tendency to allow high Na^+ or Cl^- conductance in dilute environments. For instance, transbody potentials of fish first exposed to FW are strongly negative, but within 24 h these potentials decrease to within a few mV of zero (Wood and Grosell, 2008;Wood and Grosell, 2009). The small potentials measured in acclimated fish in dilute environments suggest that there is approximately equal and low Na^+ and Cl^- permeabilities (Kirschner, 1997).

3.3 Water permeability

Osmotic permeability coefficient (P_{os}) is measured by the net rate of water gain by the whole animal or isolated gill in the presence of a transmural osmotic gradient. P_{os} represents the rate of water intake that must be balanced by fluid excretion in order to maintain in osmotic steady state; a more formal treatment appears in (Alderdice, 1988). Most studies measure P_{os} gravimetrically as mass changes, but dye dilution techniques are also used. P_{os} is distinguishable from the diffusional permeability coefficient (P_{diff}) of water movement measured by radioactive tracers in the absence of an osmotic gradient. Although the two coefficients are distinct, they are coupled and a high P_{os} would require a high P_{diff}. A low P_{os} is a critically important characteristic to isolate the animal from its osmotic environment and minimize costs of osmoregulation. Even though the gills have

a low P_{os}, the large surface area that must be available for gas exchange means that the gill accounts for the majority of the total osmotic water flow in the animal (Evans et al., 2005). The primary barrier to water permeation is the lipid bilayer and Mozambique tilapia and rainbow trout have approximately similar P_{os} in isolated gill arches in FW (Robertson and Hazel, 1999). Understandably, increasing temperature also increases P_{os} (Robertson and Hazel, 1999), suggesting lower membrane viscosity and/or higher molecular activity decreases the barrier function of the lipid bilayer. P_{os} of the whole animal however is apparently lower than that accountable by the apical membrane lipid bilayer alone, based on studies of winter flounder membrane vesicles *in vitro* (Hill et al., 2004), thus opening speculation as to other mechanisms that could contribute. Unstirred layers and tight intercellular junctions are supplementary mechanisms that could contribute to the lower P_{os} *in vivo*. Inasmuch as the apical membranes lack water channels, the basolateral membranes, particularly of ionocytes, are rich in AQP-3, a demonstrated water channel (Watanabe et al., 2005), thus volume regulatory responses of ionocytes may occur via these water channels. AQP3 is also one of the rapidly upregulated genes in several races of mummichog when these animals are challenged with 0.1 ppt FW (Whitehead et al., 2011). Tight junctions that are impermeant to water have many strands of membrane-spanning proteins and function in part to isolate apical membrane from basolateral membrane, thus excluding the AQP3 from the apical membrane. In tilapia, the tight junction protein claudin 4 and in southern flounder (*Paralichthys lethostigma*) claudins 3 and 4 are localized predominantly in the tight junctions of gill filament outer epithelial layer, and apparently more immunofluorescence signal and gene expression in FW versus SW fish (Tipsmark et al., 2008;Tipsmark et al., 2008)Claudin-8 and -27 are expressed in the skin and gill epithelia of the euryhaline pufferfish *Tetraodon nigroviridis* and moving to SW increases claudin 8c and 27b,c expression in skin and gill (Bagherie-Lachidan et al., 2009), while claudin-3 expression in gill is reduced by SW and hypersaline environment and in skin claudin 3 expression increases (Bagherie-Lachidan et al., 2008). Older research on whole body P_{os} suggested that FW fish have lower P_{os} ,(Isaia, 1984) but there needs to be a re-examination with modern techniques. The low osmotic permeability in fish gills is clearly connected to elaboration of intercellular tight junctions in the gill. It will be exciting to see the plasticity of tight junction structure be revealed in the near future.

The digestive tract also alters osmotic permeability with salinity acclimation. Whereas the esophagus epithelium is a simple columnar, osmotically permeable tissue in SW-acclimated mudskipper *Periopthalmodon modestus*, stimulated by cortisol, operating via glucocorticoid-type receptors, acclimation to FW causes epithelial proliferation and a decline in osmotic permeability, stimulated by prolactin (Takahashi et al., 2006). Mozambique tilapia has a similar esophageal response to cortisol and prolactin (Takahashi et al., 2007). In mummichog intestine where fluid absorption normally occurs, pharmacological stimulation of the epithelium via calcium and cAMP reverses fluid flow through stimulation of CFTR anion channels in the apical membrane, thus producing NaCl and fluid secretion (similar to “secretory diarrhea” in mammals typical of cholera) thus demonstrating CFTR function in intestinal NaCl transport (Marshall et al., 2002). A careful examination of polyethylene glycol (PEG) and water fluxes across intestinal epithelium of mummichogs has demonstrated that water (absorptive) flux is transcellular and not paracellular (Wood and Grosell, 2012). Transcellular water

transport involves AQP1 in European eel (*Anguilla anguilla*) intestine (Martinez et al., 2005), rather than AQP3 (Cutler et al., 2007).

Osmotic permeability of eggs and embryos is much lower than that of yolk sac larvae, gilled juveNiles and adults (Alderdice, 1988). In intertidal spawners, where terrestrial egg-laying is common, the eggs are extremely resistant to desiccation, (Taylor, 1999). Embryos of the Australian killifish (*Austrofundulus limnaeus*) have osmotic permeability 2% that of the FW zebrafish (*Danio rerio*) embryos at similar stages (Machado and Podrabsky, 2007). Embryos of mummichog also have low water permeability; measured as P_{diff}. Early pre-hatch embryos have P_{diff} of 0.4 x 10^{-6} cm/s, which is low relative to other animals but in spite of the low P_{diff} there is drinking of perivitelline fluid, apparently to maintain osmotic balance (Guggino, 1980). The low P_{diff} is consistent with a barrier function of the chorionic membrane which, in many estuarine species, has evolved to stick to vegetation and dry during development (Taylor, 1999).

4. Osmoregulatory stresses

4.1 Responses to salinity changes

4.1.1 Static changes

Transfer experiments from SW to FW and vice versa have classically examined the changes in ion and water transport and the transition from short term (min to h) coping mechanisms to long term (weeks) full acclimation responses. Estuarine fishes have evolved both and respond depending on circumstances. Many experimenters have induced euryhaline animals to acclimate to salinity change and, through these experiments, the induction of SW and FW adaptive mechanisms have been revealed, along with the hormones that affect the acclimation processes, see chapter 3 this volume (Takei and McCormick, 2013). Estuarine fishes experience a large rise in blood osmolality (60-100 mosm/kg) when transferred directly to SW; this is true for mummichog (Marshall et al., 1999;Zadunaisky et al., 1995), flounder *Paralichthys lethostigma* (Tipsmark et al., 2008), the longjaw mudsucker *Gillichthys mirabilis* and Mozambique and Nile tilapia (Velan et al., 2011) and transfer to mummichog FW produces a decrease of similar magnitude (Marshall et al., 2000), yet these imbalances are largely corrected by 24-72 hours post transfer in the new medium.

CFTR anion channel. A low conductance cAMP-activated anion channel is present in the apical membrane of SW ionocytes (Marshall et al., 1995) and the mummichog CFTR cloned and sequenced and expressed in *Xenopus* oocytes (Singer et al., 1998). CFTR is a member of the ABC (ATP binding Cassette) protein family and, unlike a typical ligand-gated channel, its gating to the open state is connected to ATP hydrolysis (Kirk and Wang, 2011). ATP hydrolysis exposes certain amino acids, Q98 in transmembrane domain one and I344 in transmembrane domain 6, in the pore of the channel (Wang and Linsdell, 2012). Acclimation of euryhaline teleosts to SW is tightly associated with increased expression of CFTR in ionocytes and onset of Cl^- secretion by opercular membrane (Marshall et al., 1999). Blockade of glucocorticoid-type cortisol receptors by RU486 impaired SW acclimation and blocked the increase in CFTR mRNA expression normally associated with acclimation to higher salinity (Marshall et al., 2005;Shaw et al., 2007), whereas blockade of mineralocorticoid type receptors by spironolactone was without effect (Shaw et al., 2007), indicating the teleost glucocorticoid-type cortisol receptor acts as an osmoregulatory mediator. Intracellular trafficking of CFTR from subapical location to the apical membrane over the first 24 h

after transfer to SW is an important means of increasing NaCl secretion rate (Marshall et al., 2002). Interestingly, a glucocorticoid-inducible kinase (SGK1) that enhances trafficking of CFTR to the cell surface is unaffected by RU486 blockade of these receptors, suggesting cortisol activation pathway of SGK1 is not via the glucocorticoid receptor (Shaw et al., 2008).

Na^+/K^+-ATPase (NKA). NKA is pivotal to ion uptake and secretion by the gill epithelium and is present in the basolateral membrane of all ionocytes. The enzyme has been cloned and sequenced from several teleost species including mummichog (Semple et al., 2002) and the gill isoform is similar to the alpha1 isoform of human. Acclimation to SW also is associated with increased expression of NKA in gill tissues of mozambique (Hwang et al., 1998;Hwang and Lee, 2007) tilapia, mudskipper (*Periopthalmus cantonesis*) (Hwang et al., 1998;Hwang and Lee, 2007) and in mummichog (Scott and Schulte, 2005).

Transfer to hypersaline conditions from SW produces a magnification of the NaCl secretion by the gill and opercular membrane and the fluid reabsorption function of the intestine, reviewed by (Gonzalez, 2012) and produces upregulation of NKA in the gill and intestine and morphological changes to ionocytes in the opercular membrane. In the Mozambique tilapia, there are separate FW and SW isoforms of NKA and the α1a isoform, supported by prolactin, decreases expression, while the α1b isoform increases expression during SW acclimation (Tipsmark et al., 2011). In SW, the intestine of mummichog normally absorbs NaCl and water (Marshall et al., 2002), although the epithelium can be induced to secrete NaCl and fluid in a response similar to secretory diarrhea. The posterior intestine of mummichog, as in most marine species, has active bicarbonate ion secretion that causes Ca and Mg carbonate precipitation in the posterior intestinal lumen and the precipitation of these solutes reduces the osmolality in the intestinal lumen, thus enhancing the continued resorption of fluid (Marshall and Grosell, 2006;Wilson and Grosell, 2003). Transfer to hypersaline environment accentuates the bicarbonate secretion in mummichog, but otherwise, there were few changes in overall function (Genz and Grosell, 2011) .

Estuarine species, on initial transfer to FW have a large decrease in plasma Na and Cl^- and osmolality, approx. 60 mosm, a decrease that is slowed down by the cessation of NaCl secretion by ionocytes in opercular membranes subjected to decrease in osmolality (Marshall et al., 2005; Acha et al., 2008;Agre et al., 2002;Bagherie-Lachidan et al., 2008;Hoffmann et al., 2007) These ion-sparing responses can be observed also as a reduction in unidirectional efflux in whole mummichog transferred to freshwater (Wood, 2011). Estuarine fishes generally survive in FW, but the salinity decrease is not necessarily connected with the development of high affinity ion uptake pumps that are typical of FW resident fish. For instance, mummichog has a K_m of 2.0 mM for Na^+ uptake across the whole body (Potts and Evans, 1967) after acclimation to FW, while rainbow trout has a K_m of 0.11 mM (Evans et al., 2005). Without a high affinity Na^+ uptake mechanism, many estuarine teleosts cannot live in ion-poor environments. For instance, gulf killifish (*Fundulus grandis*) has difficulty acclimating to salinity below 1.0 ‰ (Patterson et al., 2012).

4.1.2 Cycling salinity stresses

Recent experiments have examined the effects of cycling of temperature and salinity on estuarine species to mimic tidal cycles. These realistic experiments reveal

some novel combinations of osmoregulatory responses with stress responsesWhen mummichogs are exposed to static or cycling salinity changes while having their overall ionic permeability assessed through measurement of transbody electrical potential, these animals manifest an apparent selective permeability for cations, primarily Na^+, over anions, as they had TEP more positive than +15 mV in 100% SW, decreasing to zero mV at 20-40% SW, and more negative than -30 mV in FW (Wood and Grosell, 2009). However, mummichogs that were fully acclimated to FW showed a lower slope to the TEP change with salinity, cross zero mV at about 20% SW and had smaller positive values in seawater (+10 mV), suggesting FW acclimation produced a physiological commitment to life in dilute environment and a lower overall ionic permeability (Wood and Grosell, 2009). In mummichogs, upregulation of important regulatory genes (14-3-3 protein) (Kultz et al., 2001) and some transport associated genes, such as cell junction related genes (OCLN, CX32, CLDN3 and CLDN4) apparently lags by more than 24 h after salinity transfer (Whitehead et al., 2011), thus one would expect that major overhaul of ion transporting cells cannot occur in a transient salinity change. The core transporters, CFTR, NKA and AQP3 however, appear to upregulate mRNA expression and protein function more rapidly. CFTR in apical membrane of ionocytes is significantly upregulated (mRNA) within 8 h post transfer and Cl- secretion is elevated by 24 h post transfer (Marshall et al., 1999;Singer et al., 1998). In the case of NKA there is a rapid and transitory increase in gill NKA activity in the first few hours after the transfer of mummichogs to SW, a response that is independent of translational and transcriptional processes (Mancera and McCormick, 2000;McCormick et al., 2009). The basolateral aquaporin water channel, AQP3, is rapidly upregulated by 6 h (Whitehead et al., 2011). Thus mummichogs can rapidly modify ion and water transport function in ionocytes within hours of experiencing a new salinity, but, in some way, there is a delay in the major changes in gene expression that could lead to replacement of ion transporting cells with a new cell population. Transfer from SW to hypersaline conditions is accompanied by only slight modifications of gill (Hossler et al., 1985) and intestinal epithelia (Genz and Grosell, 2011) and electrophysiology of opercular membranes of Mozambique tilapia increase ionocyte density and decrease leak conductance (Kultz and Onken, 1993).

Transfer in the opposite direction, from SW to FW, induces in some estuarine species a novel reaction of rapid withdrawal of salt secreting cells from the surface, as has been observed independently in mudskippers, detected by CON-A fluorescence (Sakamoto et al., 2000) and in mummichogs, detected by SEM (Daborn et al., 2001). The retraction of the ionocytes is accompanied by rapid reduction in salt secretion rate and reduction in epithelial ion conductance (Daborn et al., 2001) and may be driven by a ring-like band of actin at the apical crypt opening (Daborn et al., 2001). In this way, the animals minimize ion loss on transfer to FW, even though this species does not apparently develop efficient ion uptake pathways in FW (Patrick et al., 1997;Wood and Grosell, 2009). The retraction of ionocytes to minimize ion loss in the first few hours of exposure to FW and the reversal of the transepithelial electrical potential are the likely explanation of the classically-observed “exchange diffusion effect” seen when euryhaline fishes (European flounder and mummichog) were transferred to FW and showed instantaneous reductions in Na^+ efflux across the gill (Motais et al., 1966)

4.2 Aerial exposure

In sun-exposed tide pools, resident animals can become heat stressed and hypoxic, while still needing to maintain osmoregulation. Estuarine species have evolved to cope with short time periods of osmotic and thermal stress that generally is relieved when the tide returns. Intertidal species such as blennies and mudskippers do not retreat with the ebbing tide and instead take refuge in cracks, holes and beneath rocks where they remain exposed but relatively inactive until the return of the tide. Mummichogs also survive well during aerial exposure and do not resort to anaerobic metabolism, but rather use cutaneous respiration at approximately half the usual rate of aquatic respiration in well aerated water (Halpin and Martin, 1999). This aerial exposure can result in desiccation sufficient to cause significant concentration of the blood and tissues. There is thus a trade-off between the need to maintain efficient cutaneous gas exchange, mostly across the wetted buccal epithelium (Randall et al., 2004) and the need to reduce the rate of water loss. The mudskipper *Periopthalmus cantoniensis* loses between 6 and 8% of body weight per h of aerial exposure (Gordon et al., 1978) and members of this genus die after water loss approaching 22% (Gordon et al., 1969). For four species, including Blennies *Istiblennius lineatus* and *Paralticus amboinensis* and mudskippers *Periopthalmus argentilineatus* and *Periopthalmus kalolo*, all lose water at approximately the same rate, 2.6-5.6% per h, as inanimate aqueous gel (Dabruzzi et al., 2011), implying these fish have not evolved significant defenses against desiccation. Mudskippers do not have a dry keratinized skin or waxy secretion, but there are structural modifications of the skin including increased mucus secretion and a porous corky layer that may be protective from UV light (SUZUKI, 1992). In response to high ammonia exposure, mudskipper (*Periopthalmodon schlosseri*) skin increases levels of cholesterol and saturated fats, suggestive of a protective response to reduce NH_3 permeability with the side effect of reduced evaporative water loss (Randall et al., 2004). Aerial exposure of mangrove killifish (*Kryptolebias marmoratus*) causes skin ionocytes to retract and become covered over, an effect reversible with immersion in SW (LeBlanc et al., 2010). During terrestrial sorties, interlamellar cell masses (ILCM) accumulate in the gills, an effect that may reduce evaporation on land, but also reduces effective aquatic respiratory surface area on return to the aquatic medium (Turko et al., 2011). Behaviorally, rockskippers (*Hypsoblennius gilberti*) typically emerge at night when evaporation rates are lower (Luck and Martin, 1999) and clingfish curl up near rocks to reduce the surface area available for evaporation. Mudskippers and other gobies keep their dorsal sides wet by rolling periodically (Lee et al., 2005) and restrict desiccation by spending time in their burrows (Ikebe and Oishi, 1997).

Aerial exposure also evokes changes in gill transporter distribution, an apparent evolutionary adaptation for effective excretion of ammonium ion. In mudskipper *Periopthalmodon schlosseri*, branchial ionocytes gills express NKA and NKCC on the basolateral surface, but then have NHE2, NHE3, V-type H^+-ATPase and CFTR anion channel all in the apical membrane(Wilson et al., 2000). NH_4^+ active secretion during aerial exposure results in astonishingly high accumulation of NH_4^+ in gill fluid to 90 mM in a few hours (Chew et al., 2007). In this case, the elimination of NH_4^+ may be aided by anions (HCO_3^-) permeating the CFTR pathway. Although the terrestrial sorties in mudskippers elevate plasma cortisol above resting values and cortisol actually evokes terrestrial behavior, expression of the heat shock protein HSP90 is not elevated (Sakamoto et al., 2002;Sakamoto et al., 2011).

4.3 Osmosensitivity

Osmosensitivity is reviewed in Chapter two (Kultz, 2013) ; here a few example mechanisms that control NaCl secretion rates in euryhaline teleost fish are considered. Estuarine teleosts experience large increases in plasma ions and osmolality when exposed to SW and equally large reductions in ions and osmolality when transferred to FW; these changes range to approximately 60 mosm. Ionocytes in the gills and opercular epithelium are exquisitely sensitive to small changes in osmolality, as small as 5 mosm/kg, and slight swelling of ionocytes shuts off NaCl secretion (Marshall et al., 2000), while hypertonic shock stimulates NaCl secretion (Zadunaisky et al., 1995). The effect appears to be mediated directly at the transporter level, activating NKCC in the basolateral membrane (Marshall et al., 2008;Zadunaisky et al., 1995) and CFTR in the apical membrane (Marshall et al., 2009). The activation pathway involves alpha/beta integrin as the volume sensor, several intermediate kinase steps, likely involving p38 MAP Kinase, J-N terminal kinase (JNK), Protein Kinase C, Osmotic Stress Response kinase (OSR1), ste20-Proline rich Kinase (SPAK) and Focal Adhesion Kinase (FAK) (Marshall et al., 2005). Of particular interest is FAK, that is phosphorylated at tyrosine 407 (FAKpY407) exclusively when the cells are perturbed osmotically; whereas other phosphorylation sites (Y397, Y576, Y861) are osmotically insensitive (Marshall et al., 2008;Marshall et al., 2005). FAKpY576 appears in the apical membrane colocalized with CFTR of ionocytes, while FAKpY861 is localized in the intercellular tight junctions between epithelial cells in the opercular epithelium (Marshall et al., 2008). Mummichog ionocytes are not the only fish epithelia to respond to osmotic shock by changing transport rates, as the European eel intestinal

epithelia also regulate transport rate using cell volume, reviewed by (Hoffmann et al., 2007). Whereas the osmotic responses have no effect on cAMP and are not mediated via Protein Kinase A (PKA), FAK is dephosphorylated by inhibitors and rephosphorylated at position 407 by adrenergic agonists (Marshall et al., 2009). There appears to be a multimolecular complex involving the volume sensor beta 1 Integrin, the tyrosine kinase FAKpY407 and the transporter target NKCC, colocalized by immunocytochemistry, immunogold TEM and confirmed by coimmunoprecipitation (Marshall et al., 2008). In the apical membrane, FAKpY407 also operates, but in connection with the anion channel CFTR, as FAKpY407 colocalizes with CFTR at the light and electron microscope levels by immunomicroscopy (Marshall et al., 2009). These reactions are effectively instantaneous, changing ion transport rates within a few minutes and yet the effect is long-lasting, as long as the osmotic shock is applied (Marshall et al., 2008;Marshall et al., 2005). The extreme sensitivity of this transport regulation system makes it ideal to operate in aid of estuarine fish moving between salinities for short durations, and would help them cope with salinity changes without large, metabolically expensive, acclimation processes being necessary (Marshall, 2003).

5. Estuarine fishes as physiological models

5.1 *Fundulus heteroclitus*

The mummichog is selected as a representative of an estuarine euryhaline species that fundamentally is a marine form that copes well with FW and has been an important experimental model species for over 50 years. This physiological grouping of marine-

like estuarine teleost fishes, includes mudskippers, gobies, blennies and sculpins. Mummichog are well known for their euryhaline capabilities and it can be readily acclimated to environments ranging from FW to hypersaline conditions as high as 120 ‰ (Griffith, 1974). Based upon this attribute, the mummichog has been and continues to be an important model organism for understanding mechanisms of teleost osmoregulation, as documented in two major reviews examining ionocyte structure and function (Karnaky, 1986;Wood and Marshall, 1994). Ion transport and acid-base models for teleost ion transport by gills (Evans et al., 2005;Evans, 2011) and other major osmoregulatory organs (Marshall and Grosell, 2006) also rely extensively on research with this species.

Osmoregulation in Seawater (SW) Philpott and Copeland (Philpott and Copeland, 1963) recognized numerous mitochondrion-rich cells, in mummichog gills, skin and buccal epithelium and described a curious ultrastructure, with a elaborated basolateral membrane surface in serpentine tubules that formed a ramified among the well-organized mitochondria (Philpott and Copeland, 1963). These putative ion transporting cells were similar to those seen previously in American eel (*Anguilla rostrata*) gill (Keys and Willmer, 1932). NKA, localized specifically on the basolateral membrane of these "chloride cells" (Karnaky et al., 1976) displayed higher activity in the gills of mummichogs acclimated to SW than to FW, and higher in both conditions than in fish acclimated to isotonic 1/3 SW (Epstein et al., 1967;Towle et al., 1977). The link between NKA activity and Cl^- exit from the animal into SW was a NaCl cotransporter (Silva et al., 1977) now recognized as NKCC1, a member of a larger transporter family that is rapidly phosphorylated and activated and increases gene expression during acclimation of mummichogs to SW (Flemmer et al., 2010). NKCC mediates Na^+,K^+,$2Cl^-$ transport that accumulates Cl^- above its electrochemical equilibrium inside the cell, a secondarily active transport, while K^+ recycles across the basolateral membrane via Ba^{2+}-sensitive potassium channels. An explanation for the movement of Cl^- into SW against both electrical and concentration gradients, the "pump-leak" pathway, relied on an additional discovery: an anion channel in the apical membrane that would allow the Cl^- to leak out into the environment, as summarized in Chapter 1 of this volume (Edwards, 2013) The mummichog chloride cell has a low conductance apical membrane channel that is similar to the mammalian CFTR and activated by cAMP and PKA (Marshall et al., 1995). The CFTR homologue in *F. heteroclitus*, was cloned from mummichog tissues (Singer et al., 1998) showing that its expression increased after transfer to SW, and produces a cAMP-activated anion conductance when expressed in amphibian oocytes. Acclimation to SW augments ion secretion in parallel with increased CFTR expression (Marshall et al., 1999) and there is mobilization of both the CFTR and NKCC in chloride cells, placing more of the former in apical membrane and more of the latter in the basolateral membrane (Marshall et al., 2002).

NKCC was also characterized pharmacologically. NKCC is inhibited by the "loop" diuretics bumetanide and furosemide in isolated opercular membranes of mummichog (Degnan et al., 1977;Eriksson and Wistrand, 1986) and there is dependence of NKCC on basolateral K^+ (Marshall, 2002) consistent with the Na^+,K^+,$2Cl^-$ stoichiometry. The Cl^- secretion pathway relies indirectly on the recycling of K^+ across the basolateral membrane, thus serosal Ba^{2+} that blocks K^+ channels strongly inhibits Cl^- secretion by the opercular membrane (Degnan, 1985). Also, expression of NKCC increases during

acclimation to SW (Scott and Schulte, 2005). NKCC activation involves phosphorylation (Flemmer et al., 2002), and, discovered in shark NKCC1, at three specific conserved threonine residues T184, T189 and T202 (Darman and Forbush, 2002). NKCC activation involves a suite of kinases, including ste20-related proline-alanine-rich kinase (SPAK) (Flemmer et al., 2010;Marshall et al., 2005) oxidative stress response kinase (OSR1) (Marshall et al., 2005) and a tyrosine kinase focal adhesion kinase (FAK) (Marshall et al., 2005;Marshall et al., 2008). Another kinase, with-no-lysine (WNK1) regulates NKCC in mammalian cells and in tissues from *Caenorhabdites elegans* and (Delpire and Gagnon, 2008;Kahle et al., 2010), but this needs to be confirmed for teleost fish systems. Inducible cycloxygenase, COX-2, regulates prostaglandin metabolism and mediates eicosanoid synthesis which, in turn, regulates NaCl secretion by the mummichog opercular epithelium (Evans et al., 2004;Van Praag et al., 1987).

The NKA, which is responsible for producing the Na^+ transmembrane gradient that drives NKCC operation, has also been cloned from mummichog (Semple et al., 2002). Current evidence indicates a complicated layering of regulation at the protein and gene expression levels associated with acclimation to SW. There is evidence for rapid activation of NKA within minutes, clearly derived from enzyme already in place (Mancera and McCormick, 2000). At the RNA level, NKA expression increases after transfer to SW, but interestingly is delayed by 2-3 days (Scott and Schulte, 2005). The Na^+-H^+-exchangers (NHE), which are usually associated with acid secretion in FW ion and acid-base balance (Evans et al., 2005), also appear to play a role in the regulation of acid transport in marine conditions (Edwards et al., 2005). Mummichogs drink more in seawater than in freshwater, based on sulfate and inulin radioactive markers (Potts and Evans, 1967), findings that were among the early confimations of Homer Smith's paradigm of marine fish drinking SW to offset osmotic water loss. Whereas the intestine is mostly absorptive of ions and water (Marshall and Grosell, 2006;Marshall et al., 2002) there is secretion of a carbonate rich in the posterior intestine that aids acid-base balance and water absorption (Genz and Grosell, 2011).

Osmoregulation in FW. Most of the osmoregulatory research with the mummichog has been directed toward mechanisms accommodating increased salinities, yet the converse, adjustment to lower salinities, is just as challenging and has resulted in diverse strategies (Evans et al., 2005;Marshall and Grosell, 2006). Because FW habitats are geologically transient, with inland lakes and streams forming and disappearing with geological changes, there have been innumerable opportunities for different FW osmoregulatory mechanisms to evolve (see chapter 10 of this volume, Schultz and McCormick 2013). As a result, the paradigm followed by a major model species, rainbow trout, in which acid-secreting and base-secreting cells operate in parallel to link acid-base regulation with NaCl uptake (Evans et al., 2005), is not present in many teleosts. For instance, mummichog express the pivotal enzyme V-type H^+-ATPase in the basolateral membrane (Katoh et al., 2003), rather than the apical membrane as in trout (Lin et al., 1994), implying a less direct involvement of this enzyme with ion uptake. In SW the opercular membrane secretes NaCl, as does the gill. However, in FW the gills actively absorb Na^+ but not Cl^- (Patrick et al., 1997;Patrick and Wood, 1999), while the opercular epithelium actively absorbs Ca^{2+} (Marshall, 2002;Verbost et al., 1997)(Marshall et al., 1997)and Cl^- but not Na^+(Marshall et al., 1997). Acclimation to FW relies on complex dynamics of Na^+-H^+-exchanger isoforms, NKA and carbonic

anhydrase (Edwards et al., 2005;Scott et al., 2005) and likely reflects both a rapid turnover of transporting cells (Daborn et al., 2001;Laurent et al., 2006) and a Na^+ uptake system that could instead rely on Na^+-H^+ exchange or neutral NaCl cotransport. As a result, Na^+ uptake is much less efficient in mummichog than in the trout, yet it is a mechanism that still operates sufficiently to allow mummichog to occupy even ion-poor FW habitats, so long as the animals are actively feeding. Indeed, the ionic contribution of food is essential for mummichogs to survive in ion-poor FW because Cl^- uptake is limited in the opercular membrane and is absent from the gill (Laurent et al., 2006;Marshall et al., 1997).

Kidney and Intestine. In the marine environment, mummichogs drink to offset osmotic water loss to the environment (Potts and Evans, 1967) in a reflex stimulated by angiotensins (Malvin et al., 1980). The intestine absorbs water and ions from the SW ingested by the animal and in the posterior portion there is also some ion secretion, specifically of bicarbonate (Marshall and Grosell, 2006). The digestive tract of the mummichog is unusual, as it lacks a stomach and is relatively short. The intestine is normally absorptive, involving NaCl absorption in parallel to water channels, presumably AQP1, as discovered in European eel (*Anguilla anguilla*) (Martinez et al., 2005) that impart hydraulic conductivity and allow fluid reabsorption. The kidney of teleosts is an important means for excretion of water by FW-acclimated teleosts and in marine teleosts for the secretion of divalent ions, especially Mg^{2+}, Ca^{2+} and SO_4^{2-} (Marshall and Grosell, 2006). The mummichog kidney is small and has no urinary bladder, where ureteral urine might accumulate and be altered before release. In SW, tubular secretion of Na^+ and Cl^- occurs as secondary active Cl^- transport with electrically coupled paracellular Na^+ transport. Electrochemical gradients for K^+ and Na^+ allow Cl^- entry across the basolateral membrane presumably *via* NKCC and results in cytosolic Cl^- concentrations above electrochemical equilibrium. Apical Cl^- secretion is *via* a cAMP stimulated conductive pathway, by an as yet unidentified anion channel (Marshall and Grosell, 2006). In both SW and FW killifish, NKCC immunoreactivity was detected in the apical membrane of the distal and collecting tubules, and in the basolateral membrane of the second segment of the proximal tubules, the major part of killifish renal tubules (Katoh et al., 2008). These results support the secretory function of the early and absorptive function of the more distal portions of renal tubules of these euryhaline fishes. In FW, the killifish proximal tubule is also secretory (Beyenbach and Liu, 1996), being balanced by reabsorptive NaCl transport in the distal portions of the tubule for the net excretion of a dilute urine. Study of the spatial and temporal expression of transporters in the kidney would reveal the dynamics of this tissue during salinity changes. Development of miniaturized techniques and molecular approaches that use small tissue samples will enable future studies of transporter and channel expression in the kidney, a wide-open and particularly fascinating area for study.

Although the important discovery of CFTR in fish occurred in mummichog, and other transport protein genes are now known (NKA, NHE, carbonic anhydrase, H^+-ATPase, NKCC), many other transporter homologs (*e.g.* K+ channels, AE1, NCC, Ca-ATPase and CLC channels) and their regulatory proteins remain enigmatic. To date four potential regulatory proteins have been sequenced, CFTR itself (Singer et al., 1998), the glucocorticoid receptor (Mommsen et al., 1999), COX-2 (Choe et al., 2006) and the 14-3-3 protein (Kultz et al., 2001)

5.2 *Oreochromis niloticus*

The Nile tilapia (*Oreochromis niloticus*) is a rapidly-growing, omnivorous FW and estuarine species, closely related to a FW euryhaline teleost model species Mozambique tilapia (*O. mossambicus*). Nile tilapia is subject to worldwide aquaculture exceeding 2.0 million tonnes as of 2007 (FAO statistics) and occupies brackish lagoons of the Nile delta (Oczkowski and Nixon, 2008) and estuaries of Africa, Indonesia, China, the Caribbean and South America. It is FW tolerant and can even acclimate to deionized FW (Pisam et al., 1995) and to salinities up to 60 ppt (Guner et al., 2005;Schofield et al., 2011). Because the species is tropical, salinity acclimation ability is better in warm (30 ^{o}C) water and colder water temperatures limit survivability in temperate SW (Schofield et al., 2011). The gill epithelial structure indicates pavement cells interrupted by exposed ionocytes, mucous and pillar cells (Monteiro et al., 2010).

Freshwater (FW). Acclimation to deionized water evokes elaboration of the microvilli (Velan et al., 2011) on the apical membrane of ionocytes, suggestive of increased area for ion uptake proteins (Pisam et al., 1995). The ion uptake mechanism in the Nile and Mossambique tilapias involves the NaCl cotransporter NCC expressed in the apical membrane of gill ionocytes (Velan et al., 2011) as well as basolateral NKA and NKCC1. If the relatively low affinity (a few mM) NCC is the main NaCl uptake mechanism, it is not clear how these animals survive in deionized water, unless this environment invokes an as yet undiscovered high affinity Na^+ uptake mechanism. It may be dietary ions act as a supplement. In any case, the NCC and low ion permeability are supported by high expression of PRL1, the gene for prolactin synthesis (Breves et al., 2011).

Seawater (SW). Because of the critical importance of Nile tilapia aquaculture utilizing more estuarine habitats, salinity tolerance of various tilapia species and hybrids among tilapia species (Fall et al., 2011;Wang et al., 2000) has attracted much interest (Guner et al., 2005;Rengmark et al., 2007;SURESH and LIN, 1992). These fish have a "U" shaped NKA activity relationship with salinity, in that NKA activity is high in very dilute environments, low in brackish conditions and high again in SW and hypersaline conditions up to 60 ‰ (Guner et al., 2005). Transfer from FW to 15 ppt brackish water downregulates NCC and prolactin (PRL1) expression in Nile tilapia, while Mozambique tilapia had this reaction plus a strong immediate upregulation of NKCC and NKA, yet growth hormone expression was apparently unchanged in both species (Velan et al., 2011). Nile, During the acclimation to hypersalinity, Nile and Mossambiqyue tilapia develop unusually large multicellular ionocyte complexes in the gills (Figure 4) (Cioni et al., 1991;Guner et al., 2005). The multicellular complexes appear to comprise multiple accessory cells and one large ionocyte (Fontainhas-Fernandes et al., 2003). Four genes that have been identified as important in SW acclimation and growth in Nile tilapia are hemoglobin, Ca^{2+}-ATPase, pro-opiomelanocortin (POMC) and actin (Rengmark et al., 2007). Hyperosmotic transfers of Nile tilapia also increase expression of osmotic stress factor 1 (OSTF1), prolactin 1 (PRL1), growth hormone (GH) and branchial GH receptor, while branchial expression of the ion uptake transporter Na-Cl cotransport (NCC) is downregulated and NKCC expression is unchanged after six h (Breves et al., 2010). The slow response of Nile tilapia to increasing salinity may be part of a larger adaptive pattern for estuarine species to cope with short term changes and to react to permanent changes some 24 h later.

5.3 Tolerance vs. Acclimation

Full acclimation to new salinity regimes is performed rapidly in estuarine animals and they usually tolerate direct FW-SW and SW-FW transfers, unlike diadromous species. Estuarine fish combine the short term coping strategies with long term acclimation to effect a full plasticity in osmoregulation (Marshall, 2003;Wood, 2011). It may be that euryhaline estuarine fish have evolved a means of delaying expression of stress responsive proteins such as 14-3-3 that are expressed in killifish transferred to FW only after 24 h (Kultz et al., 2001). NKA expression also is delayed by 2-3 days after transfer to SW (Scott and Schulte, 2005) . By delaying onset of large, energetically expensive remodeling of tissues, the tolerance strategy would save metabolic energy. Similarly, the SW type of cation permeability that produces the large negative-inside transbody potential in mummichog is not lost for 24 h in FW, an effect thought to contribute to their ability to return to SW quickly (Wood and Grosell, 2008). When challenged by 0.1 ppt salinity, mummichog populations native to brackish or marine habitats lose osmotic homeostasis, more severely and take longer to recover compared to freshwater populations, and although AQP3 expression responds quickly, many other genes respond more slowly (Whitehead et al., 2011). In a group with FW, estuarine and marine members, the sculpin genus *Cottus,* FW and marine-resident species transferred to SW immediately initiate strong drinking response, whereas the estuarine species has much lower drinking rate even 24 h after transfer (Foster, 1969). Rapid small changes in osmoregulation involve the reversible retraction of ionocytes from the surface on exposure to hypotonic media or hypotonic shock has been observed in mudskippers (Sakamoto et al., 2000) and in killifish (Daborn et al., 2001). In mangrove killifish (*Kryptolebias marmoratus*) ionocytes retract during aerial exposed sessions (LeBlanc et al., 2010). Emergence of these cells again is triggered in mudskippers by external SW or high calcium (Sakamoto and Ando, 2002). Transfer of climbing perch to SW initially evokes high blood sodium, to 170 and 164 mM in 1/3 and full strength SW, that is corrected by seven days in SW, accompanied by a rise in NKA activity in the gills (Chang et al., 2007). Nile tilapia transferred to SW for 6 h show no increase in NKCC expression (Breves et al., 2010), yet this species can acclimate to twice SW salinity, if salinity is increased gradually (Guner et al., 2005). Transfer of mummichogs to SW also elicits rapid (~ 8 h) increases in CFTR (Marshall et al., 1999) and NKCC expression (Flemmer et al., 2010) and trafficking of CFTR to the apical crypt where NaCl secretion is increased by 24 h (Marshall et al., 1999). There is also evidence for rapid activation of NKA and corresponding ion fluxes apparently without the need for protein synthesis (Mancera and McCormick, 2000;Wood, 2011). Therefore, rapid upregulation and activation of a small cadre of genes seems to be the only major change that is necessary to initiate full SW level NaCl secretion by ionocytes of the gill epithelium, rather than proliferation and differentiation of ionocytes that characterizes long-term acclimation.

Similarly, tide pool and estuarine species may be more tolerant of temperature fluctuations and tolerate change rather than react to it. Tide pool fish are exposed to large environmental fluctuations; for example tide pool sculpins have evolved higher tolerance to temperature shifts and their Heat Shock Protein (HSP) responses are activated at higher temperatures than their subtidal equivalent species (Nakano and Iwama, 2002). This may result from the animals in fluctuating conditions having higher resting levels of HSPs. Whereas the means by which estuarine fish delay the large tissue turnover

acclimation steps, from a variety of perspectives, the tolerance strategy seems to fit the combined results of many transfer experiments.

Hemoconcentration during terrestrial sorties by tropical intertidal mudskippers is minimized behaviorally by these animals. Mudskippers roll in the mud to reduce evaporative water loss (Ikebe and Oishi, 1997). They also will return to their water-filled burrows, a behavior driven hormonally by 11-deoxycorticosterone, apparently operating through mineralocorticoid type receptors, an effect partially blocked by the steroid receptor blocker RU-486 (Sakamoto et al., 2011). Behavioral solutions to osmoregulatory responses also are economical and do not require large physiological adjustments.

Exposure to hypotonic conditions of intertidal blennies causes behavioral changes consistent with a tolerance response. Upper intertidal black pricklebacks (*Xiphister atropurpureus*) consume O_2 at a significantly lower rate in dilute SW, compared to full-strength SW, whereas there was no significant difference in O_2 consumption by penpoint gunnels (*Apodichthys flavidus*) that frequent lower intertidal tide pools (Haynes et al., 2009), suggesting the hypometabolic tolerance response to salinity change, rather than acclimation, in animals exposed to more environmental variability.

5.3 Genomics and proteomics

The estuarine fish genomes available are expanding and several euryhaline species genomes are complete or almost so (Fugu *Takifugu nigroviridis* , threespine stickleback *Gasterosteus aculeatus* and mummichog *Fundulus heteroclitus*) so that there will be more access to full genomic models in the near future (Table 1). Given the huge commercial importance and deep EST file, it is surprising that Nile tilapia has attracted less scientific interest than its cousin Mozambique tilapia (ESTs submitted = 355). Scientists could do well to shift attention to Nile tilapia, the most aquacultured euryhaline teleost on the planet, and a critical protein source for many developing nations. These genomic advances will allow full molecular manipulation experiments to be performed, thus opening new and exciting possibilities for understanding the acclimation processes to various combinations of stressors often faced by estuarine species. The studies will be made all the more interesting because of the ancient total genomic duplication event in the actinopterygian fish lineage (Cutler and Cramb, 2001;Larhammar et al., 2009). The biomonitoring function of the estuarine genomic fish model *Fundulus heteroclitus* has been demonstrated in a recent study examining salinity gradients and the effects of salinity variation in the habitat with genetic variation in the important clusters of osmoregulatory genes (Whitehead, 2010;Whitehead et al., 2011). With careful and ongoing documentation of the genomic database (Paschall et al., 2004) comparative transcriptomics can reveal essential functional genes that allow the northern subspecies of mummichog to be stronger hypoosmoregulators. Key to effective hypoosmoregulation in FW is reduction in passive permeability through development of effective tight junctions in skin and gill epithelia, supportive calcium metabolism and rapidly responsive (6 h) osmotic stress factors (OSTF1) and aquaporin3 (AQP3) genes (Whitehead et al., 2011) that help maintain junctional integrity and enhance basolateral hydraulic conductivity in aid of cell volume regulation. A few well-placed trancriptomics studies provide many physiological questions that can be answered using genomic techniques, such as selective knockout and knockdown approaches to revealing functional importance of certain genes. For instance, morpholino knockdown techniques have been developed in mummichog to test cytochrome P450-1A (CYP-1A) in examining the role of this enzyme in

detoxification (Matson et al., 2008) and, in zebrafish, to discern the function of Rhcg1 in ammonium and Na^+ transport (Kumai and Perry, 2011) Importantly, multiple genes of unknown function will be highlighted and subsequently their functions can be revealed.

6. Conclusions and the future

From this discussion emerges a trend toward a tolerance strategy used by some estuarine fishes, wherein the animals cope with short term salinity (and other) changes without launching full salinity-acclimating mechanisms and only invoke long term acclimation mechanisms after a delay of a day or more. The mechanism(s) controlling this delay are currently unknown. This tolerance strategy is shared by semiterrestrial estuarine fishes that cope with hemoconcentration from evaporative water loss during terrestrial sorties. Estuarine fish, in coping with frequent salinity change, use osmosensitive ion transport cells that respond to slight changes in environment and blood to shut off ion secretion and to retract the cells below the epithelia surface to protect from excessive ion loss in FW. Estuarine fish use many adaptive behaviors and salinity preferences to minimize their exposure to large salinity changes. Estuarine fish will continue to be important model species that are representative of most osmoregulatory strategies of the teleosts. The completion of important genomes will allow more sophisticated and probing questions to be asked. Remaining unresolved mechanisms include the structure and function of paracellular pathways in FW an SW, the interactions between the various transporters, especially post-translational variations and regulation by kinases, the role of NHE and Rh proteins, and further resolution of the Na^+ uptake channel. There needs to be more microarray studies to discover the important regulatory factors for the ion transporters and water channels. Future studies may also focus on behavioral osmoregulation at the population extreme and molecular regulatory responses at the reductionist extreme, to complete the picture of adaptation to a continually changing environment.

Figure Legends

Figure 1. Profile through intertidal zone at high tide (upper panel) and at low tide (lower panel). Hatched underwater area is full strength SW below the halocline. Circular arrows indicate weak mixing above the halocline. Note that there are zones, indicated along the bottom of the figure, where a locus may be exposed to SW only (subtidal), SW alternating with FW, and FW only, depending on the elevation above tide datum. The tide pool in the estuary may fill with SW on a large tide, brackish water on a moderate tide or be inundated by rain and fill with FW at low tide. During large tides and with strong winds, mixing can be higher amplitude and disrupt stratification, producing uniform brackish mixed salinity (not shown).

Figure 2. Upper panel: A) Presentation of theoretical Nernst potentials $\{E_x = (RT/z_iF) \ln (C_{io}/C_{ii})\}$ for $x = Na^+$ and Cl^- plotted against the isosmotic/isoionic conformational line, which, in this log-linear plot, is a curve asymptotic to the abscissa. The two E_{Na} and E_{Cl} lines and the isoionic line cross zero mV at an external Na^+ of approximately 155

mM, isoionic to the blood plasma. Note that the measured TEP in isoionic to hyperionic (1/3 SW and above) salinities approximates the slope of the Na^+ equilibrium potential line but is above by 10-20 mV (the result of active Cl^- secretion), effecting a consistent voltage gradient favoring cation (Na^+) secretion via a cation selective paracellular pathway. The transbody potential measured *in vivo* approximates E_{Na} in iso- and hypertonic saline. In more dilute environments, the TEP deviates from the Na^+ equilibrium potential (presumably because of less cation selectivity) and has a polarity that would aid cation uptake, but is insufficient alone to produce cation uptake. The TEP is even farther from the Cl^- equilibrium potential, demonstrating that anion uptake occurs against a steep electrochemical gradient. Whereas some estuarine teleosts have effective NaCl uptake (*Oreochromis niloticus*) others, *e.g. Fundulus heteroclitus* simply fail to generate anion active uptake at the gill and substitute Cl^- uptake at the gill by anion absorption via the intestine. In hypersaline conditions, both *Fundulus* (Genz and Grosell, 2011;Marshall et al., 1999;Scott et al., 2006) and hybrid tilapia (Garcia-Santos et al., 2006;Gonzalez, 2012;Wang et al., 2009) sharply increase plasma Na^+ in parallel to the isoionic line, as they reach their physiological limit.

Lower Panel B) Depiction of the transmembrane concentration gradients in FW and SW ionocytes and the re-expression of the fold-gradients as Nernst equilibrium potentials. Intracellular ion activities for ionocytes have not been measured and are estimates based on other NaCl secreting epithelia (corneal epithelium and airway). In two cases, Na^+ in FW and Cl^- in SW, the equilibrium potential for the apical membrane approximates a normal negative-inside cellular electrical potential, thus in these two cases, Na^+ uptake and Cl- secretion across the apical membrane could be via ion channels (driven by the action of other active transporters). In SW, the apical CFTR anion channel is the pathway for Cl- secretion (Marshall et al., 1995;Singer et al., 2008) and in FW the recently-discoveredacid sensing ion channels (ASIC) (Chen et al., 2007) are Na^+ channels that, by knockdown and pharmacological experiments with zebrafish (Dymowska personal communication), are possible candidates for the Na^+ uptake channel. .

Figure 3. Plot of blood versus environmental variables for osmoregulation of a series of Blenniid species of intertidal teleosts, two of which survive in hypersaline conditions (> 1100 mosm/kg) but none of which survive in extremely dilute (< 10 mosm/kg) FW. Superimposed is data from the Nile tilapia, *Oreochromis niloticus*, that can survive in ion-poor environments, even to deionized water (Fall et al., 2011;Velan et al., 2011). In hypersaline conditions, plasma osmolality rises sharply in parallel to the isosmotic line as the fish approach their upper physiological limit (see also Figure 2a).

Figure 4. Multicellular mitochondria rich cell complexes that develop in the gill epithelia of Nile tilapia, *Oreochromis niloticus,* in full strength SW when the fish are slowly acclimated to increasing salinity (Cioni et al., 1991). These complexes have numerous accessory and ionocytes congregated about a single large apical crypt and the gill has lower numbers of these but higher NKA activity (Guner et al., 2005).

Table 1 Genomes of osmoregulatory model species and related commercial importanc

Trivial Name	Scientific Name	Source (NCBI)	#Total EST	Habitat	*Aquaculture	*Fishery
Zebrafish	*Danio rerio*	RefSeq	1,773,474	Freshwater stenohaline	0	0
Japanese pufferfish	*Fugu rubripes*	Ensembl	Sanger Inst. 2002 Aparicio et al. 2002	Marine stenohaline	?	?
Greenspotted pufferfish	*Tetraodon nigroviridis*	GenBank	Jaillon et al. 2004	Estuarine euryhaline	?	?
Atlantic salmon	*Salmo salar*	dbEST	498,212	Anadromous	1,440,085	?
Channel catfish	*Ictalurus punctatus*	dbEST	354,488	Freshwater stenohaline	449,753	1,000
Rainbow trout	*Oncorhynchus mykiss*	dbEST	287,967	Freshwater euryhaline	732,432	(Incl)
Threespine stickleback	*Gasterosteus aculeatus*	dbEST	276,992	Estuarine euryhaline	0	0
Atlantic cod	*Gadus morhua*	dbEST	255,256	Marine stenohaline	12,000	900,000
Blue catfish	*Ictalurus furcatus*	dbEST	139,475	Freshwater stenohaline	(incl in channel catfish)	
Nile tilapia	*Oreochromis niloticus*	dbEST	120,991	Estuarine euryhaline	2,542,960	200,000
Mummichog	*Fundulus heteroclitus*	dbEST	90,441	Estuarine euryhaline	0	0
Gilthead sea bream	*Sparus aurata*	dbEST	74,877	Marine euryhaline	124,000	?
Common carp	*Cyprinus carpio*	dbEST	35,347	Freshwater stenohaline	3,216,203	?

#Total Expressed Sequence Tags filed to NCBI July 20 2012

*FAO United Nations 2009, tonnes.

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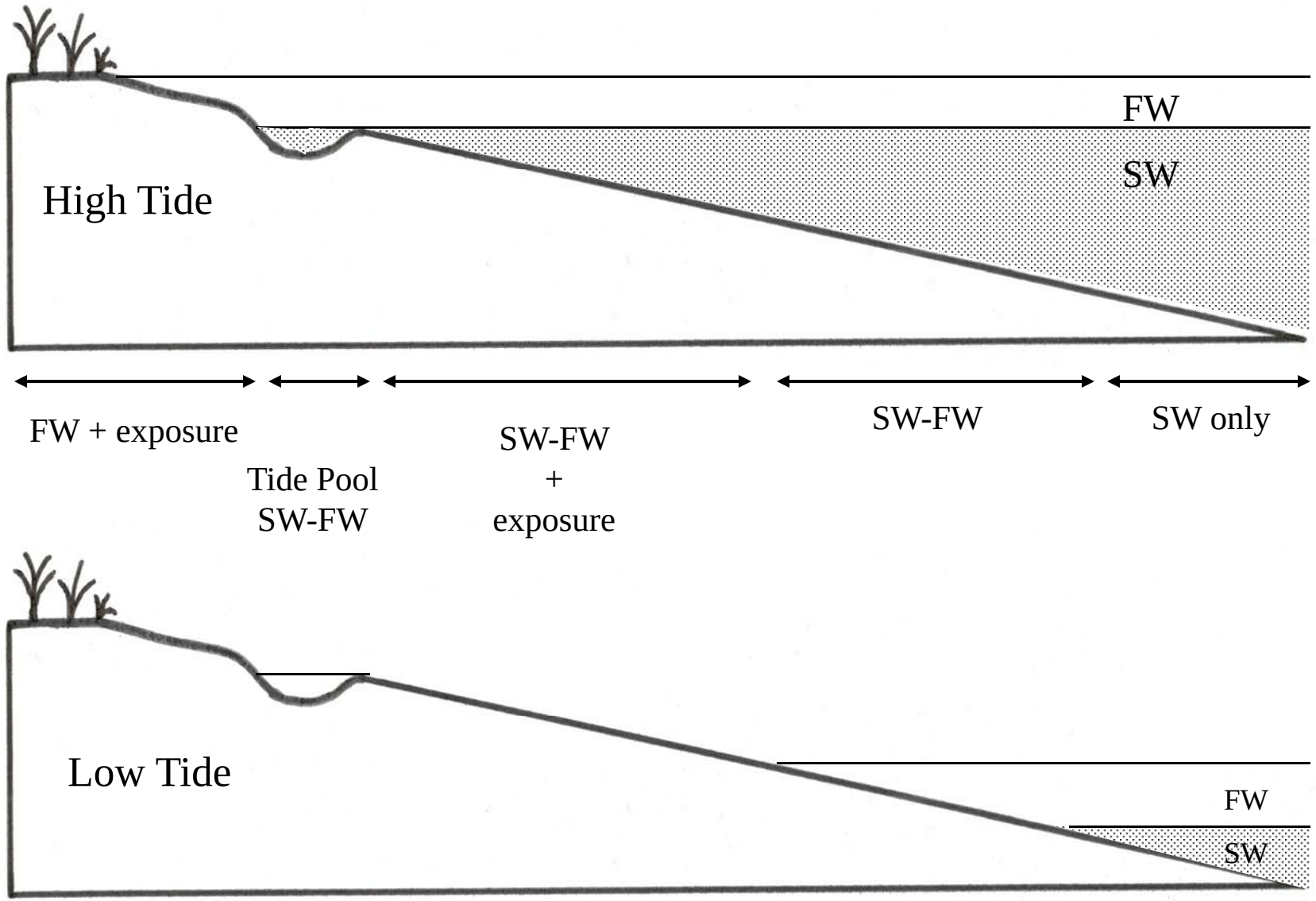
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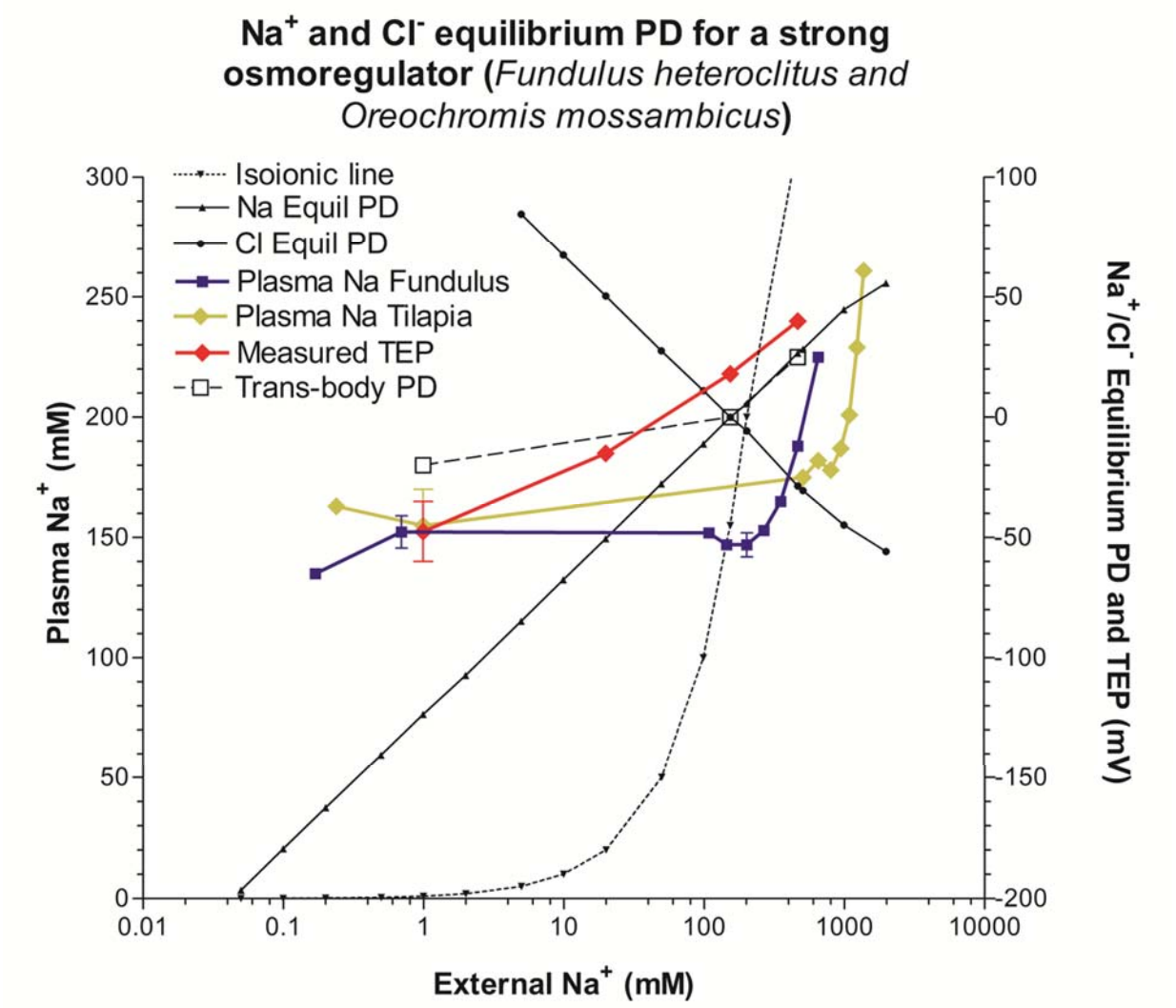
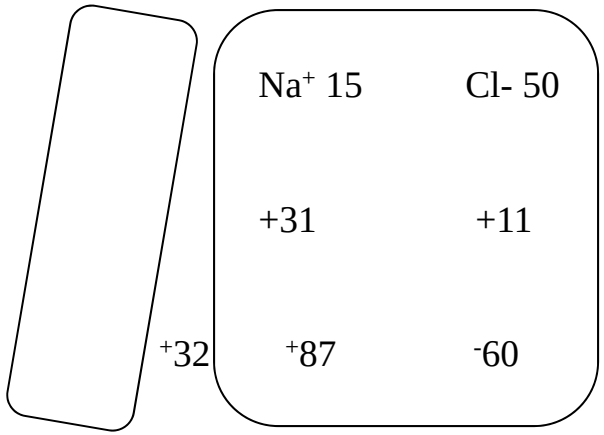
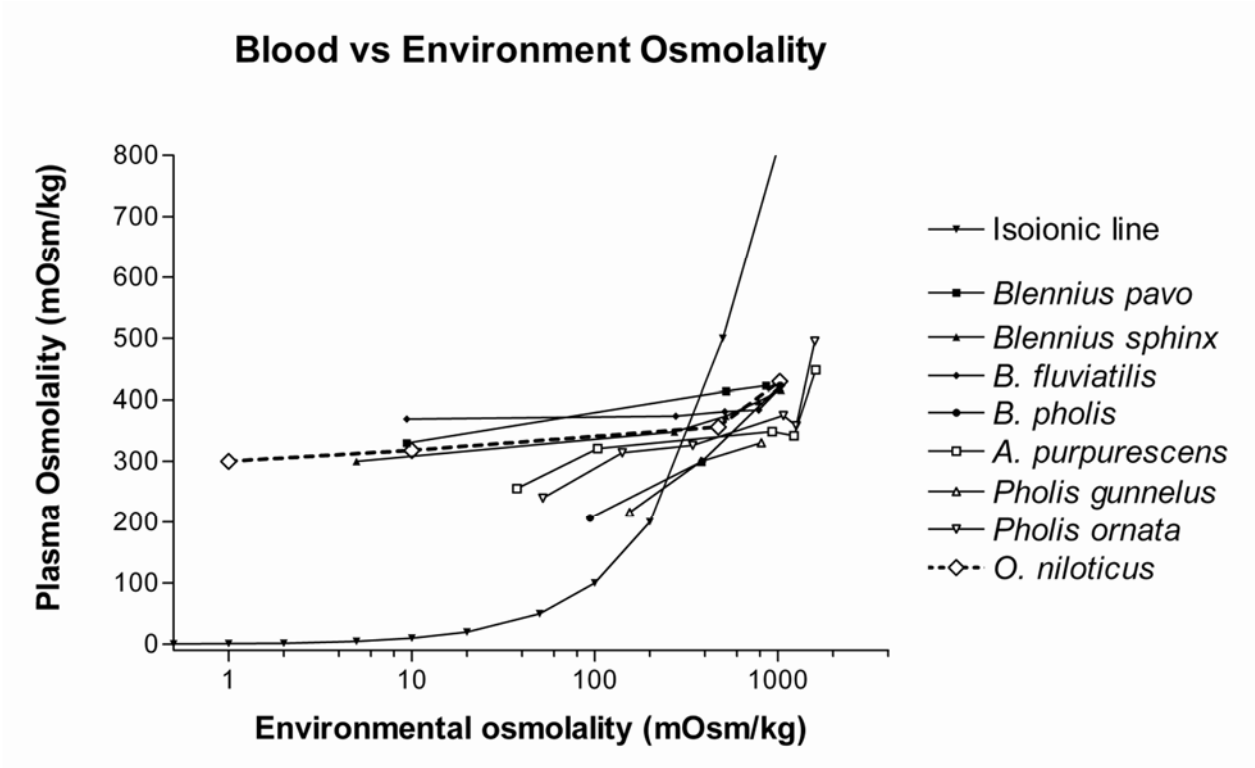
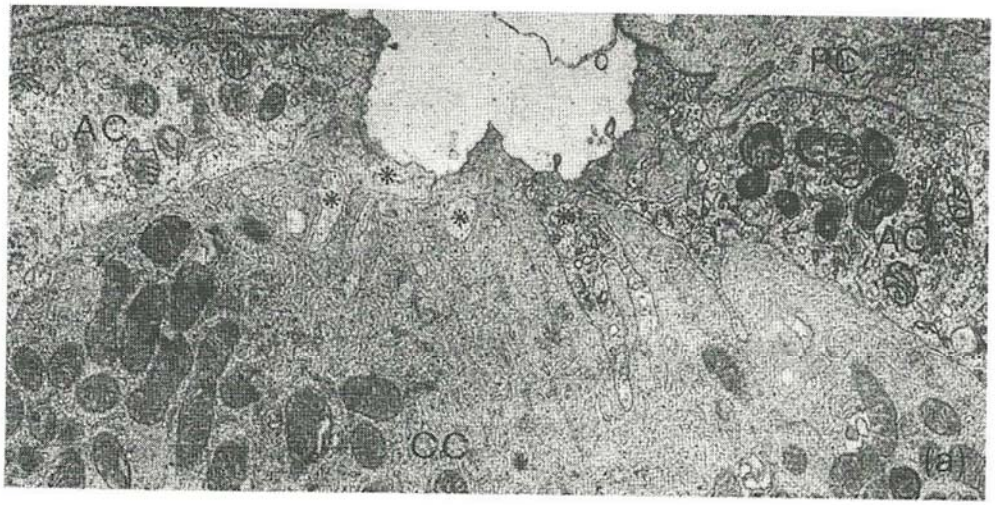

Na+ and Cl- equilibrium PD for a strong osmoregulator (Fundulus heteroclitus and Oreochromis mossambicus)
Isoionic line
Na Equil PD
Cl Equil PD
Plasma Na Fundulus
Plasma Na Tilapia
Measured TEP
Trans-body PD
Plasma Na+ (mM)
Na+/Cl- Equilibrium PD and TEP (mV)
External Na+ (mM)
300
250
200
150
100
50
0
100
50
0
-50
-100
-150
-200
0.01
0.1
1
10
100
1000
10000

	Freshwater		Seawater	
Environment Ions (mM)	Na^+ 0.1-1.0	Cl- 0.1-1.0	Na^+ 469	Cl- 546
Intracellular Ions (mM)	Na^+ 15	Cl- 10	Na^+ 15	Cl- 50
Fold-Gradient	$^-15$ - $^-150$	$^-10$ - $^-100$	+31	+11
As Equil. PD (mV)	$^-68$-$^-126$	$^+58$-$^+116$	$^+32$	$^+87$ $^-60$
Extracellular ions (mM)	Na^+ 140	Cl^- 130	Na^+ 155	Cl^- 145




Blood vs Environment Osmolality
Plasma Osmolality (mOsm/kg)
800
700
600
500
400
300
200
100
0
1
10
100
1000
Environmental osmolality (mOsm/kg)
Isoionic line
Blennius pavo
Blennius sphinx
B. fluviatilis
B. pholis
A. purpurescens
Pholis gunnelus
Pholis ornata
O. niloticus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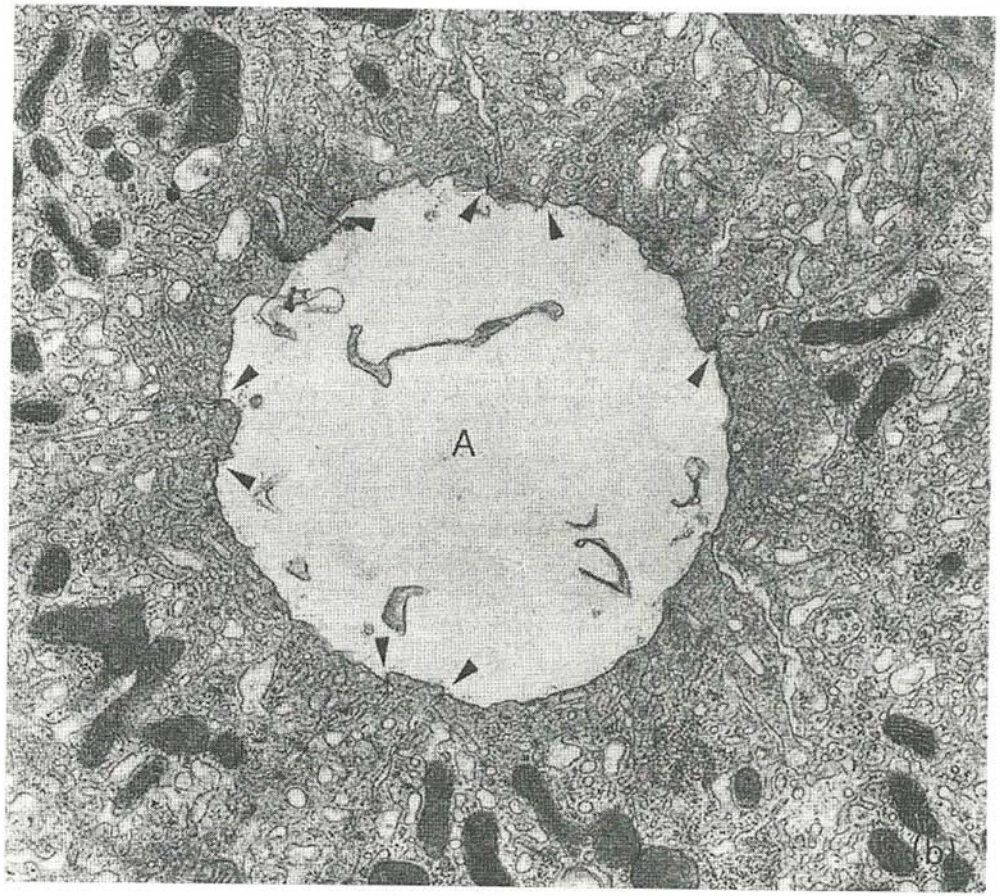
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