https://doi.org/10.47886/9781934874615.ch9

Chapter 9

Osmoregulation and Acid-Base Balance

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9.1 TOPIC BACKGROUND

9.1.1 Basic Concepts in Osmoregulation and Acid-Base Regulation

Maintaining relatively constant levels of internal cellular ions is critical to the normal function of all animals. For many organisms this is achieved primarily by regulating the ion and acid-base composition of the blood within narrow limits. This understanding of the importance of "le milieu interior," first espoused by Claude Bernard in the mid-1800s and later described as "homeostasis" by Walter Cannon, is a cornerstone of modern physiology. "It was Bernard's view that we achieve a free and independent life, physically and mentally, because of the constancy of the composition of our internal environment" (Smith 1961:1). Direct contact between the gills and water makes ion, water, and acid-base balance especially challenging and important to fish and, in turn, makes fish important subjects for understanding the evolution and control of all of these homeostatic processes.

Several strategies exist within fishes for regulating ion concentrations in the blood relative to external (environmental) salt concentrations. Hagfishes, which are one extant group representing the ancestral jawless condition of vertebrates, are restricted to seawater (SW) and have an osmoconforming strategy in which the internal (blood) and external osmotic concentrations are very similar (Currie and Edwards 2010), but important differences do exist (Sardella et al. 2009). Lampreys are the other group of extant jawless fishes and either live wholly in freshwater (FW) or are **anadromous**. Lampreys have an osmoregulatory strategy in which the internal concentrations of ions are approximately one-third that of SW (Reis-Santos et al. 2008). Their underlying mechanisms of ion transport and osmoregulation appear to be nearly identical to those of the more recently evolved ray-finned fishes (Figure 9.1), which have adopted a similar osmoregulatory strategy. Elasmobranchs and coelacanths in SW retain high levels of urea in their plasma and are osmoconformers (Figure 9.2), whereas in the relatively rare instances elasmobranchs are found in FW, they are hyperosmoregulators, maintaining plasma ion levels in excess of environmental levels via mechanisms similar to FW ray-finned fishes (Ballantyne and Robinson 2010).

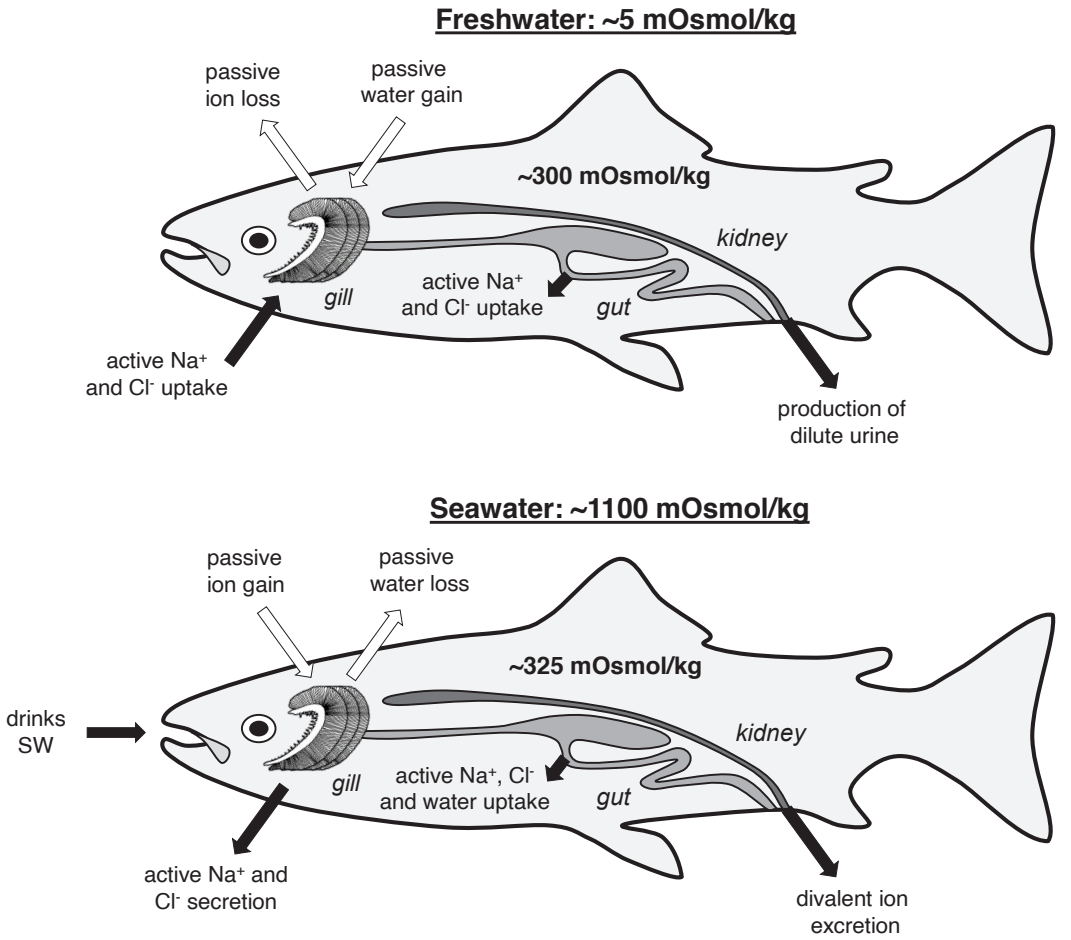

Figure 9.1. Diagram of passive (light arrows) and active (dark arrows) salt and water movement in freshwater and seawater in teleosts. The gill, gut, and kidney are the primary organs involved in ion regulation in juvenile and adult teleosts, with the skin performing the function of the gill in larval stages. Approximate total osmotic concentration in freshwater and seawater and internal osmotic concentration are presented as mOsmol/kg. Figure created by Daniel Hall and Stephen McCormick.

Fish with an osmoregulatory strategy must counteract passive forces of water and ion flux in both FW and SW in order to maintain a constant internal osmotic concentration of one-third SW (Figure 9.2). In FW, fish face diffusional loss of ions and gain of water across all surfaces in contact with the external environment, primarily the gills and skin. To counteract these passive forces, they take up Na^+ and Cl^- across the gills or from food across the gut and produce a copious, dilute urine (Evans et al. 2005). In SW, fish face diffusional loss of water and gain of ions. Fish drink when in SW, both water and ions are absorbed by the gut, followed by secretion of monovalent ions (Na^+ and Cl^-) by the gill and excretion of divalent ions (Ca^{2+} and Mg^{2+}) by both the intestine and kidney (Evans et al. 2005). Approximately 48% of all fish are restricted to marine environments and 48% restricted to FW environments (Betancur-R

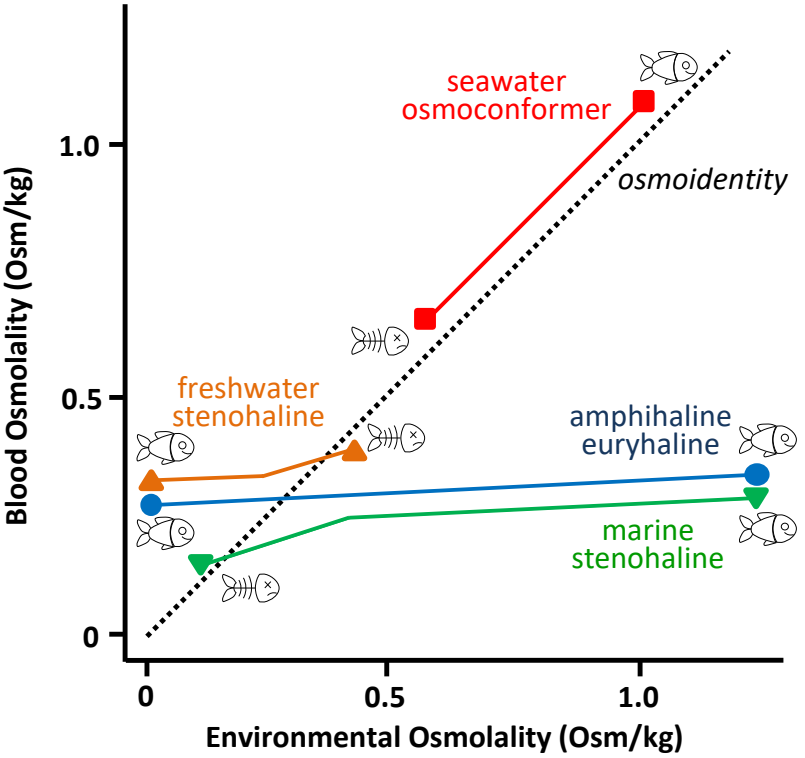

Figure 9.2. Plasma osmolality (Osm/kg) in relation to environmental salinity in fish with different osmoregulatory strategies. Modified from Marshall and Grosell (2006) and created by Daniel Hall and Stephen McCormick.

et al. 2015). The restriction in **halohabitat** may reflect a restricted **halotolerance** and a limited ability to switch between the mechanisms for active uptake of ions necessary in FW and active secretion of ions necessary in SW. Only about 4% of fish species are **amphihaline**, consisting primarily of intertidal, estuarine, or migratory (diadromous) species that undertake migrations between FW and SW (Schultz and McCormick 2013). **Euryhaline** species have the capacity to maintain internal (blood) ion concentrations relatively constant irrespective of the salt concentration of their external environment. Responses of blood osmotic concentration of **stenohaline** and euryhaline lampreys and ray-finned fishes following abrupt changes in external salinity are shown in Figure 9.3.

Blood acid-base status in fishes is tightly regulated and is integrated into the ionoregulatory system. Deviations in pH affect all levels of biological organization, from protein structure and function to the whole organism. In the absence of regulation, acid-base status would be affected by everything from metabolism to more severe disturbances that can arise internally (i.e., exhaustive exercise, low blood O_2 [**hypoxemia**]) and externally (i.e., low environmental O_2 [**hypoxia**], low pH [see Box 9.1], elevated CO_2). Blood acid-base balance is maintained by equimolar exchange of acid-base relevant ions (i.e., Na^+/H^+ exchange, Cl^-/HCO_3^- exchange) with the environment, predominantly at the gills in both FW and SW fish (Heisler 1986; Ev-

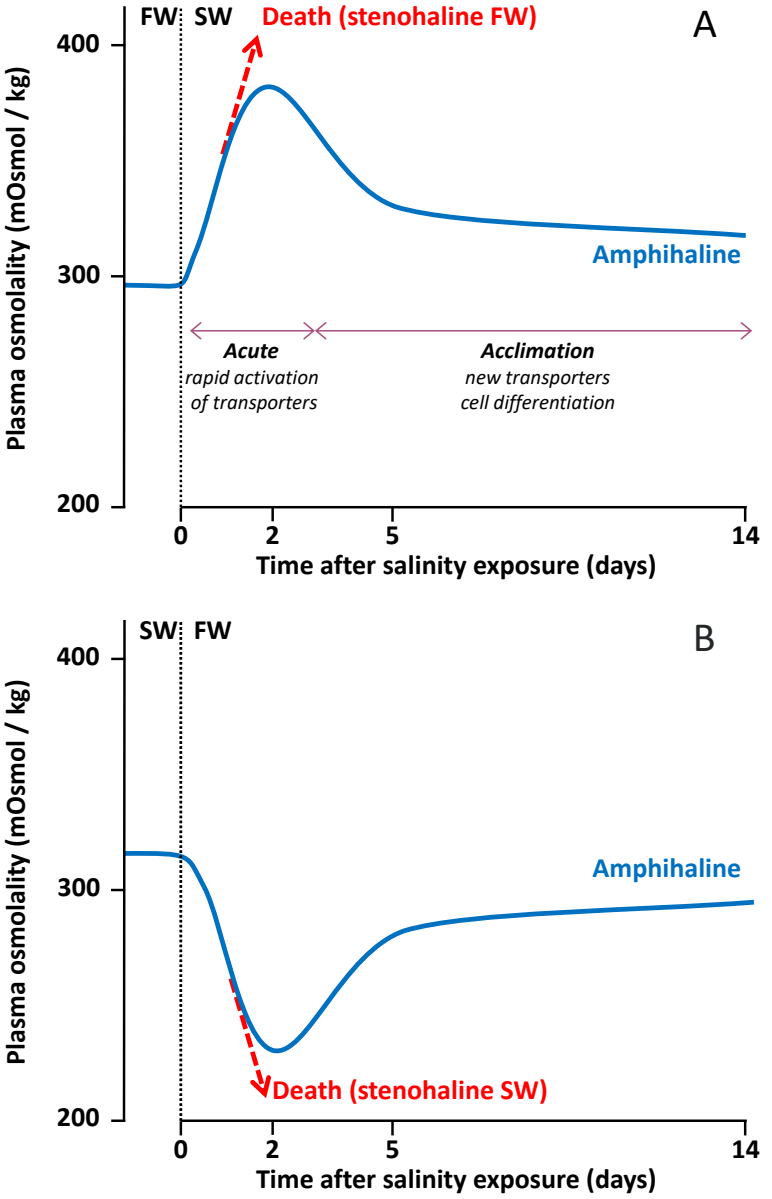

Figure 9.3. Time course of changes after (A) freshwater (FW) to seawater (SW) and (B) SW to FW transfer in stenohaline and euryhaline fishes. Two different phases have been described in euryhaline fishes after transfer between water of different salinities: an "acute" phase lasting from 1 to 3 d and an "acclimation" phase from 3 to 14 d (Holmes and Donaldson 1969). These phases may reflect the rapid activation and/or recruitment (insertion into membranes) of existing transporters in the acute phase and the production of new ion transporters and ionocytes in the regulatory phase (McCormick and Bradshaw 2006). Figure created by Daniel Hall and Stephen McCormick.

Box 9.1 Case Study: Acid Rain and Acidification

As we have described, fish have evolved complex physiological systems to tightly regulate osmoregulatory and acid-base status and maintain homeostasis. However, conditions today often differ markedly from the environments in which fish evolved. Understanding the mechanisms and thresholds of osmoregulation and acid-base balance permits us to predict and possibly mitigate the effects of environmental change. For example, the devastating effects of acid rain on fish in the 1970s, especially in low Ca^{2+} waters, were connected to the displacement of Ca^{2+} from the tight junctions of gills by H^+ and aluminum (the latter made more toxic at low pH), leading to a lethal loss of osmoregulatory performance (Milligan and Wood 1982). The negative effects were ameliorated by adding Ca^{2+} ("liming") to lakes and rivers. Thus, understanding the mechanism of impairment facilitated some measure of mitigation.

Concern about the consequences of elevated atmospheric CO_2 levels, including ocean acidification (Heuer and Grosell 2014) and much-less-studied freshwater acidification (Ou et al. 2015; Hasler et al. 2016), have renewed interest in acid-base regulation in fishes, building on the foundational work of the 1970s and 1980s (Heisler 1986). Another impetus for renewed study of acid-base regulation is the increasing use in aquaculture of recirculating systems and closed containment. Such systems enable higher culture densities but also require supplemental O_2 that can lead to greatly elevated CO_2, which in turn decreases pH. The regulation of acid-base balance influences fish growth, development, and welfare under these poorly understood conditions (Brauner and Richards 2020).

ans et al. 2005). Thus, pH regulation is intimately associated with an animal's ion regulation. Blood pH values are relatively uniform among fishes (but vary predictably with temperature), whether they are euryhaline, stenohaline FW or SW, osmoconformers, or osmoregulators. Even hagfishes have a very efficient response to deviations in blood pH that is based upon equimolar exchange of acid-base relevant ions at the gill (Baker et al. 2015).

Metabolically produced CO_2 diffuses across the gill, where the partial pressure of CO_2 (PCO_2) in the blood equilibrates with water. The control value (time 0 h) presented in Figure 9.4 represents a typical resting value for a water-breathing FW fish at 15°C. For this temperature in fish, blood pH values typically range from 7.8 to 8.0, PCO_2 values from 2 to 4 mmHg and plasma HCO_3^- from 5 to 8 mM. If environmental PCO_2 increases, blood PCO_2 increases and blood pH is reduced (as CO_2 readily dissociates to HCO_3^- and H^+) along the blood buffer line (Figure 9.5), referred to as a respiratory acidosis. In a longer-term exposure (24–96 h) to elevated CO_2, blood pH recovers along the respective PCO_2 isopleth (Figure 9.4; metabolic alkalosis), predominantly through processes at the gills (see section 9.2.4) that result in a net increase in plasma [HCO_3^-] in exchange for [Cl^-].

In addition to regulation of plasma levels of ions and acid-base status relative to the external environment, there is a second level of regulation between the plasma and all cells. The osmolality of plasma and cells are the same, but the ion levels in cells are different from the plasma. Cellular [Na^+] is lower, and [K^+] is higher, than in the plasma because of the action of Na^+/K^+-ATPase (NKA). Cells also have a greater abundance of dissolved organic compounds such as amino acids, making total ion content and pH within cells lower than in the plasma.

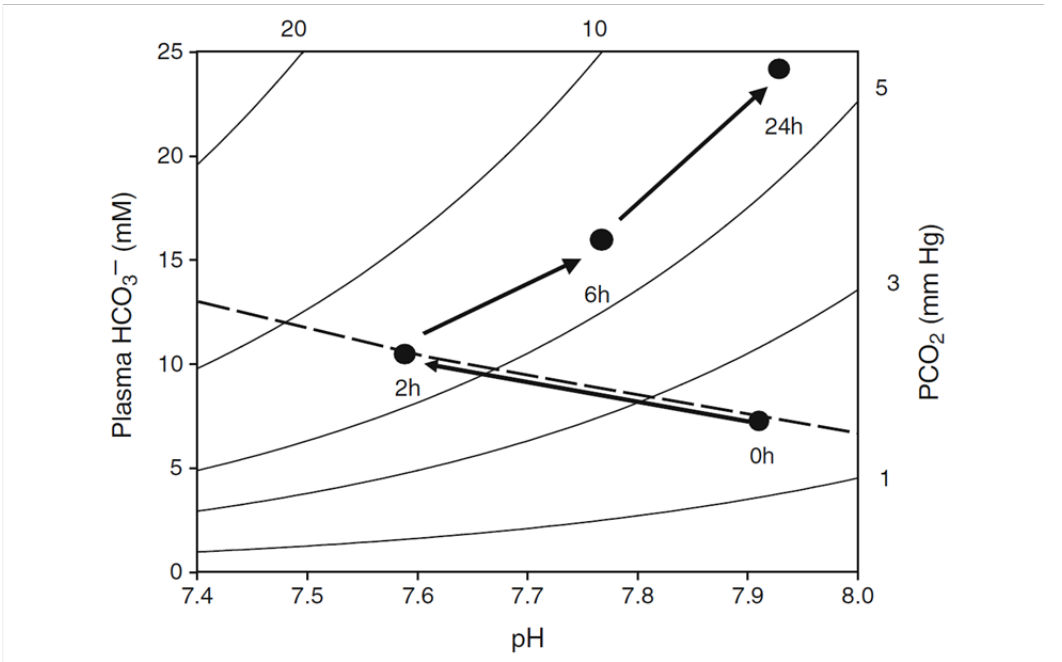

Figure 9.4. The effect of sustained elevated water PCO_2 (partial pressure CO_2, mm Hg) on blood pH and plasma HCO_3^- in a freshwater Rainbow Trout *Oncorhynchus mykiss* over time. Isopleths represent the constant PCO_2 values indicated and the dashed line indicates the blood buffer line. Numbers on data points indicate times of exposure to elevated CO_2 and the associated pH compensation (data replotted from Larsen and Jensen 1997).

Free calcium in the plasma is regulated very tightly at around 1 mM but is much lower within cells (0.1 μM), and most calcium in cells is sequestered in organelles (Moon 1994).

Responses to changing salinity include changes in the abundance in fish tissues of small organic molecules called osmolytes that function in maintaining cellular osmolality (Edwards and Marshall 2013). Even in osmoregulating organisms, cells must osmoconform because there is no means of maintaining an imbalance in overall solute concentrations across the cell membrane. Hence, changes in body fluid osmolality will induce efflux or influx of cellular water and potentially a dangerous decrease or increase in cell volume until osmolyte concentrations are adjusted. Osmolytes are classified as compatible if their abundance has little effect on cellular biochemistry; compatible osmolytes include amino acids such as taurine. Perturbing osmolytes such as urea have a tendency to destabilize or denature proteins or DNA, and counteracting osmolytes such as trimethylamine oxide ameliorate the effects of perturbing osmolytes.

Hormones are important in many aspects of homeostasis, including ion, water, and acid-base balance of fishes. By definition, hormones are released into the blood and have actions in a variety of specific target tissues. Hormones act on specific receptors that are located on the cell membrane, intracellularly, or in the nucleus of target tissues, depending on the specific hormone. Cortisol, growth hormone, and prolactin are directly involved in the regulation of ionocytes and their ion transporters (Takei and McCormick 2013). A variety of other hormones rapidly regulate drinking (the renin-angiotensin system) and ion transport (arginine vasotocin, natriuretic

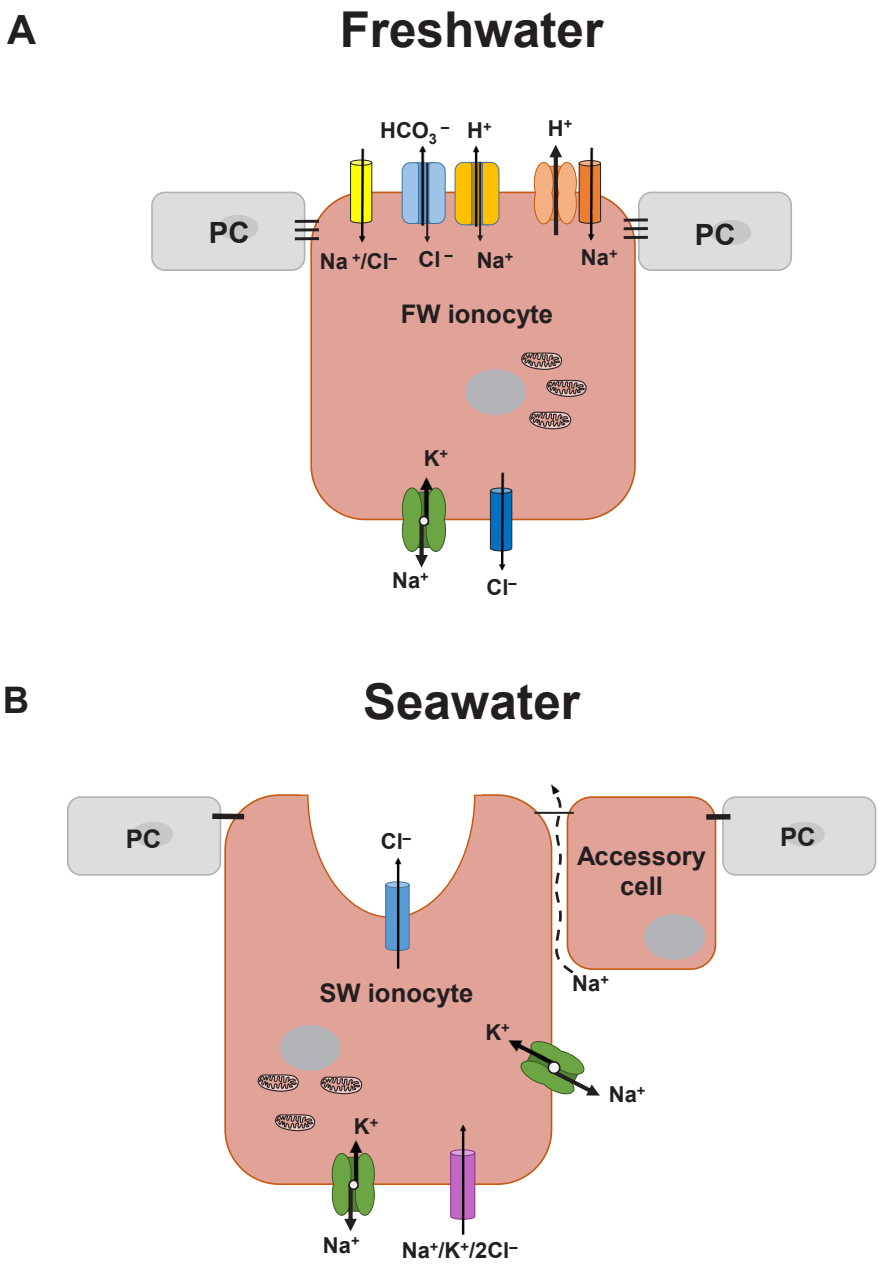

Figure 9.5. Schematic of ion transporter in gill of fish in (A) freshwater and (B) seawater. Some apical ion transporters shown in freshwater gill ionocytes may also be located in pavement cells (PC). Figure created by Diogo Ferreira-Martins and Stephen McCormick.

peptides, urotensins). The basic endocrinology of fishes and other aspects of their participation in salt and water balance can be found in Jeffrey et al. (2022, Chapter 14, this volume), and practical aspects of sampling and manipulation later in this chapter (section 9.2.5).

9.1.2 Gill and Ionocyte Function

As mentioned above, the gill is the dominant surface for osmoregulation and acid-base status; it is also the primary surface for O_2 uptake, CO_2 excretion, and nitrogenous waste removal. Thus, it is a truly multifunctional organ (Evans et al. 2005). However, with all these competing physiological processes come trade-offs in terms of optimizing gill design. Features that maximize gas exchange (a high surface area and thin diffusing distance) make it more difficult to iono- and osmoregulate. Thus, the characteristics of the gill will differ depending upon the environment in which a fish lives and the specific challenges it may face. For example, during hypoxia fish rapidly increase ventilation rate to enhance O_2 uptake. However, in a few species prolonged exposure to hypoxia results in gill remodeling that results in longer and thinner respiratory lamellae (Sollid et al. 2003; Matey et al. 2008). These responses increase the effective surface area of the gill and reduce blood–water diffusion distance, thus increasing passive ion and water effluxes and compromising plasma ionoregulatory status, illustrating what is known as the osmo-respiratory compromise (Sardella and Brauner 2007; Gilmour and Perry 2018).

The gill is the primary site to overcome passive ion diffusional forces, taking up Na^+ and Cl^- in FW and secreting them in SW (Figure 9.1; Evans et al. 2005; Edwards and Marshall 2013). This function is carried out by specialized cells called **ionocytes**, also known as mitochondria-rich cells or chloride cells (Hiroi and McCormick 2012). These cells have an extensive tubular system, continuous with the basolateral membrane, containing transport proteins that translocate ions. Transporters are membrane-bound proteins that are critical to the net movement of ions and water. Active transporters can move ions against their concentration gradient. Primary active transporters bind and hydrolyze ATP to power the transport of ions against their electrochemical gradients. Some of the most important of these in fish are Na^+/K^+-ATPase, H^+-ATPase, and Ca^{+2}-ATPase. Secondary active transporters do not directly use ATP; rather, they harness the potential energy of electrochemical gradients produced by primary active transporters to move other ions upgradient. Secondary active transporters are symporters, which transport two or more ions across the cell membrane in the same direction (the NKCC1 in the basolateral membrane of SW ionocytes is a prominent example), or are antiporters, which move one ion downgradient and another ion upgradient and across the membrane in the opposite direction (e.g., the gill Cl^-/HCO_3^- exchanger). Finally, some transporters conduct only facilitated diffusion (downgradient cross-membrane transport), such as transport of water by aquaporins.

Freshwater ionocytes generally have a broad apical surface with microvilli that contain apical ion transporters (Figure 9.5A). Sodium-hydrogen exchange (often involving a Na^+ channel coupled to H^+-ATPase) and Cl^-/HCO_3^- exchange occur at the apical surface, moving Na^+ and Cl^- into the cell (Edwards and Marshall 2013). In some species there is also an apical NaCl cotransporter (NCC) that is involved in ion uptake (Hiroi et al. 2008). Then, Na^+ and Cl^- are moved across the basolateral membrane into the blood by NKA and a Cl^- channel, respectively. In some species these functions appear to occur in a single ionocyte, but in other species specialized ionocytes have evolved; Zebrafish *Danio rerio* have five

different types of FW ionocytes with distinct functions for exchange of Na^+, Cl^-, Ca^{2+}, and acid-base balance (Hwang et al. 2011).

Seawater ionocytes are generally larger than FW ionocytes, and unlike the latter usually make direct contact with both the external environment and the blood (Figure 9.5B). They have a deep apical crypt with a small opening. On the basolateral membrane, NKA transports three Na^+ outward and two K^+ inward, thus creating both an ionic and electrical gradient (McCormick 1995). A basolateral $Na^+/K^+/2Cl^-$ (NKCC) cotransporter then "drags" two Cl^- and one K^+ into the cell using the favorable sodium gradient. Then Cl^- leaves the cell through the apical cystic fibrosis transmembrane regulator (CFTR) on a favorable electrical gradient. The Na^+ ion is secreted through a paracellular pathway, the specificity of which is likely determined by tight junction proteins between ionocytes and adjacent accessory cells.

9.1.3 Drinking and Gut Function

In addition to the roles it plays in digestion, nutrient absorption, and disease resistance, the gut also functions in regulation of water and ion balance of teleost fish. In FW, the gut of feeding animals can absorb significant levels of Na^+ and Cl^- even against the prevailing concentration gradient (Bucking and Wood 2006). Uptake of Ca^{2+} by the gut is tightly regulated in both FW and SW (Marshall and Grosell 2006). In SW the gut has the critical role of absorbing water that is passively lost to the hypertonic environment (Figure 9.1). Seawater exposure rapidly induces increased drinking rates, which are generally 10-fold higher than those seen in FW. Water cannot be transported selectively against its concentration gradient, and the process of water absorption can occur only after the gut first absorbs ions to the point that gut fluid is hypotonic to the blood. The esophagus has been shown to absorb ions in some fish species (Hirano 1976), but it is unclear whether this is universal among fishes. Further absorption of ions occurs in the intestine through the action of an apical NKCC and basolateral NKA. The gut further facilitates water uptake via secretion of HCO_3^- into the lumen; the decrease in pH precipitates $CaCO_3$ and $MgCO_3$ and thereby reduces gut fluid osmolality (Wilson et al. 2009). The consequent reduction in blood pH enhances O_2 unloading from hemoglobin to this metabolically active tissue (Cooper et al. 2014) but creates an acid-base disturbance that must be corrected at the gills. Precipitation of $CaCO_3$ and $MgCO_3$ also facilitates excretion of divalent ions that would otherwise be partially absorbed and then need to be cleared by the kidney.

Elasmobranchs in SW are osmoconformers but ionoregulators; high levels of blood urea require that they secrete Na^+ and Cl^-, which is conducted by a specialized organ in the gut called the rectal gland. It is composed of glandular arrays of mitochondria-rich cells that are structurally similar to the gill ionocytes of ray-finned fishes and use the same transporters (basolateral NKA and NKCC and apical CFTR) for ion secretion.

9.1.4 Kidney and Urinary Bladder Function

The kidney of fishes has important functions in overall water and ion balance. In FW, the kidney and urinary bladder produce a bountiful and highly dilute urine, thus ridding the body of passively gained water. Most FW fishes have a glomerular kidney that provides an initial filtrate (Hickman and Trump 1969). Urine flow rate is tightly connected to total filtration rate (Marshall and Grosell 2006), which is determined by the number of active nephrons (Brown

et al. 1980). Reabsorption of ions occurs mostly in the distal tubule and additionally in the proximal tubule and collecting duct. In many species additional ion reabsorption occurs in the urinary bladder. While the gill is the dominant site for acid-base regulation (90%), the kidney does play a minor role, most notably in HCO_3^- reabsorption (Heisler 1986).

In SW, the kidney produces an isosomotic urine and thus is only indirectly involved in water balance. Seawater fishes commonly have aglomerular kidneys or reduced numbers of glomeruli (Hickman and Trump 1969). The rate of urine production in SW fishes is greatly reduced, around one-tenth that of FW fishes. Aglomerular kidneys produce urine at a rate comparable to glomerular kidneys, using tubular secretion rather than filtration (Marshall and Grosell 2006). Secretion of divalent cations and regulation of Ca^{2+} are an important function of the SW fish kidney, which produces urine that is relatively low in $[Na^+]$ and high in $[Mg^{2+}]$, $[SO_4^{2-}]$, and $[Cl^-]$. At present no readily detectable markers of response to changes in environmental salinity are known for the kidney that are comparable to those in the gill, such as changes in ionocyte morphology and levels of NKA, NKCC, and CFTR.

9.2 TECHNIQUES/METHODS

9.2.1 Halotolerance

A common method for determining halotolerance is acute (or direct) exposure to one or more salinities (Schultz and McCormick 2013). This method is akin to that commonly used in ecotoxicology, but in contrast to tests of environmental toxicants, tolerance limits are associated with a dearth as well as a surfeit of salt. Following direct transfer from an acclimation salinity to one of multiple levels of test salinity, subjects are scored for an endpoint (usually mortality) at intervals for a prescribed period that is shorter than the timescale of an acclimation response. Subjects are typically not fed in direct treatments to maximize control over salinity and water quality and to minimize variability among individuals within a group. Results of such experiments are often plotted as survivorship trajectories for each test salinity over time, showing the time course of the proportion of individuals that reached the endpoint. By convention, the tolerance limit is defined as the LC50, the median or mean salinity at which 50% of individuals have succumbed at a specific time point, but an alternative such as the LC10 might be estimated if the salinity conferring a higher rate of survival is of interest (e.g., for aquaculture; Faulk and Holt 2006). The LC50 can be roughly estimated via interpolation between two survival rates at the specified time point; for example, if survivorship at 48 h was 40% at 10 ppt (parts per thousand) and 60% at 14 ppt, LC50 would be estimated as 12 ppt. This informal approach uses only a small subset of the data from a lethal dose trial and cannot support inference tests. A formal approach using all the data from a trial is therefore preferable; it requires modeling the probability of succumbing as a function of salinity and time by means of logistic regression (e.g., Faulk and Holt 2006), probit regression (e.g., Shahriari Moghadam et al. 2013), or a nonparametric procedure (e.g., Bringolf et al. 2005). Experimental design considerations in direct transfer tests include the number of individuals per experimental unit, replication of experimental units (e.g., tanks), the number of salinity treatment levels, the interval at which endpoints are checked, and the total time of the trial. For larval stages, the trial period is often less than 24 h because mortality rates are high under any conditions; for juvenile and adult fish, trials typically last 3 or 4 d.

Other methods include testing halotolerance endurance, meaning the limit that is reached after the ability to acclimate is exhausted, rather than the ability to tolerate acute change. Subjects undergo a stepped series of salinity changes (e.g., 1 ppt per day); the stepped changes are paced to remain within the subject's ability to acclimate, at least initially. Unlike the direct transfer method, the gradual method requires feeding so that the result is unambiguously a response to salinity rather than starvation. Endpoints are monitored over time; in addition to monitoring survival, growth or reproduction might also be measured. Halotolerance may be quantified by estimating the salinity at which 50% of individuals in each experimental unit succumbed. A related approach that has been used to inform niche prediction modeling conducts gradual transfers to various fixed salinity levels that are maintained while monitoring for multiple fitness metrics (e.g., Lowe et al. 2012). Experimental design considerations in gradual transfer tests include the number of individuals per experimental unit, replication of experimental units, and the schedule (including both timing and magnitude) of salinity changes. Halotolerance studies using the gradual method have not adopted a standard schedule of salinity changes; one recent experiment on juvenile Threespine Stickleback *Gasterosteus aculeatus* increased salinity by 2 ppt every day (Divino et al. 2016). Feeding subjects introduces complications. Care should be taken to ensure that salinity does not diverge from desired levels because of food addition, and if live food is used it must be able to tolerate the full range of test salinity levels at least briefly. Because food is a source of ions, the observed level of halotolerance may be affected by the food source, and differences in feeding rate among individuals may magnify variability in halotolerance.

Whether by the direct or gradual transfer method, salinity tolerance tests generally employ mortality as the endpoint. However, measurement of changes in plasma $[Na^+]$, $[Cl^-]$, and osmolality as described for the "24 hour SW challenge test" have been used to provide sublethal indicators of salinity tolerance in fish (see Box 9.2) and are described in more detail below (section 9.2.3). Experiments that test tolerance to temperature or oxygen tensions often score for loss of equilibrium or other sublethal indicators (e.g., McDonnell et al. 2019), with the benefits of reduced use of animals and/or opportunities for additional physiological measurements at the endpoint. Sublethal behavioral endpoints have rarely been used in salinity tolerance testing (but see Young and Cech 1996; Verhille et al. 2016), which suggests that the neuromuscular system mediating commonly used sublethal endpoints in thermal and hypoxia tests are less affected by loss of osmoregulatory homeostasis.

9.2.2 Blood Sampling

The way in which blood is sampled is important for measurement of osmoregulatory parameters and is crucial for acid-base status. Ion levels and osmolality change slowly relative to the time needed to sample, and thus blood can be obtained on euthanized fish via various procedures (discussed in greater detail below). To prevent coagulation, blood is usually collected into a heparinized syringe or vial, subject to several considerations: heparin can interfere with some plasma hormone assays, and if plasma $[Na^+]$ is to be measured, ammonium-heparin should be used rather than sodium-heparin. Also, EDTA has been used as an anticoagulant but is less widely used because of its apparent impact on pH and PCO_2 when used at high concentrations (Houston 1990). The blood should be spun down at sufficient revolutions per minute to produce 3,000 g-force units for 5 min to separate plasma from cells. Plasma can be frozen, preferably at –80°C until analyzed. Dehydration is a concern when very small plasma volumes are stored for long periods of time.

Box 9.2 Case Study: Salmon Aquaculture and Smolt Studies

Commercial aquaculture for salmon is currently valued at more than US$10 billion per year. This industry began in the 1960s and grew slowly, in part because of a poor appreciation of physiological changes that naturally occur in the life history of these fishes. Salmon undergo a transformation from the stream-dwelling parr to the downstream migrating smolt that comprises morphological, physiological, and behavioral preparations for ocean entry (Hoar 1988; McCormick 2013). Improper conditions (such as small size or improper photoperiod and/or temperature cues) in the months prior to normal smolting resulted in poor survival and growth upon transfer to seawater (McCormick et al. 2009). Owing to the need for a relatively simple, ethical, and repeatable test of smolt development, Craig Clarke and colleagues developed the "24 hour SW challenge test" (Clarke 1982). This method relies on the fact that plasma ions generally peak 24 to 48 h after transfer from FW to SW (usually 35 ppt). In parr, which have relatively poor salinity tolerance, plasma [Na^+], [Cl^-], and total osmolality increase by 30%, whereas in smolts the increase is less than 10%. The "24 hour SW challenge test" could be conducted on a much smaller number of test subjects than would a survival assay to assess salinity tolerance and doesn't require mortality as an endpoint, which is ethically questionable on a large scale. This measure of salinity tolerance was shown to increase in spring in smolts and correlate with increased long-term survival and growth in SW (Clarke 1982).

Another test of readiness for SW focuses on a pathway that enhances salt secretory capacity. Wally Zaugg developed a relatively simple procedure to monitor gill Na^+/ K^+-ATPase (NKA) activity and showed that it increases 5- to 10-fold in salmon smolts relative to parr (Zaugg 1982). Refinements of the technique include the use of gill biopsies for nonlethal testing (McCormick 1993a), which can be advantageous especially when sampling from endangered populations. The SW challenge combined with measurements of gill NKA are methods in wide use in monitoring the extent and time course of development of salinity tolerance in smolts and together provide a comprehensive assessment of salinity tolerance. See Clarke et al. (1996) for a more detailed discussion of the trade-offs and practical aspects of monitoring smolt development. Future advances may involve measurement of the SW-specific form of NKA, which increases dramatically during smolt development (McCormick et al. 2013). Monitoring smolt physiology can be useful to ensure that hatchery conditions in both conservation and commercial settings yield fish with robust smolt development as well as to determine the effect of altered environmental conditions on wild salmon (Brauner and Richards 2020).

A different sampling approach is needed for analysis of acid-base status. Blood pH changes rapidly as PCO_2 rises (see Figure 9.4), as could occur when fish struggle during capture. The preferred method to sample fish blood for acid-base analysis is through an indwelling catheter at least 24–48 h after implantation (Soivio et al. 1975). If cannulation is not possible, blood may be drawn from the caudal vessels. In this case, blood should be drawn as soon as fish have been anaesthetized using buffered tricaine methanesulfonate (MS-222). Gills should be ventilated or at least immersed in water while blood is withdrawn to minimize disturbances to acid-base status (Brauner et al. 2019). Blood pH values will fall if the process takes longer

than a few minutes, and this may account for low pH values measured by clinical analyzers (see section 9.2.4). Once blood is collected into a heparinized syringe, it should remain sealed and held on ice for no more than a few minutes prior to measurement.

The caudal blood vessels and the heart are the main sites for collecting single samples of blood (Black 2000). Caudal blood can be accessed by puncture with a hypodermic needle on a ventral or lateral approach posterior to the anal fin (e.g., Behrens et al. 2017) or with a heparinized capillary tube following caudal transection (e.g., Kusakabe et al. 2017; Kolbadinezhad et al. 2018). Larger blood volumes can potentially be collected by puncturing the ventricle with a hypodermic needle on a ventral approach (e.g., Kijewska et al. 2016). With the latter approach the area to be cut should be blotted dry first to minimize contamination by surface water. One comparison of methods found no difference in blood osmolality between blood sampled at the heart and the tail (Marino et al. 2001). However, Marino et al. (2001) and others (e.g., Samaras et al. 2016) have demonstrated that blood osmolality can be affected by stress arising from conditions of confinement and handling. Plasma ions are not affected by whether the subject is sacrificed immediately prior to blood sampling, nor whether blood is collected by caudal puncture or cannulation of the dorsal aorta. They can be affected by exchanges between the plasma and erythrocytes, and blood should therefore be centrifuged as soon as is practical and certainly before 8 h after collection (Clark et al. 2011). Additional considerations and background for blood collection can be found in Eliason et al. (2022, Chapter 10, this volume).

9.2.3 Plasma Osmolality and Ions

The movement of water across a membrane separating two fluids, such as a cell membrane or the gill epithelium separating the environment from body fluids, depends on the relative concentration of solute particles in the two fluids. The concentration of solute particles, quantified as osmolality, cannot be calculated for most solutions because the extent to which molecules have dissociated into separate particles cannot be derived analytically. Solute concentration is instead measured in complex solutions such as plasma as one of several colligative properties relative to standard solutions. Nordlie (2009) reviewed analyses of teleost body fluid osmolality to ascertain sources of variability across life history characteristics and broad habitat classifications.

One such colligative property that can be measured on plasma is vapor pressure, since water has a lower tendency to evaporate from solutions with higher concentrations of solutes. Vapor pressure osmometers control and sense the temperature of a thermocouple hygrometer in a sample chamber. Via a cycle of voltage changes, the hygrometer quantifies dew point in the sample chamber relative to that of the operating environment; there is a direct relationship between dew point depression and vapor pressure in the chamber relative to saturation. Once the sample is introduced into the chamber, the process that yields a calibrated reading of osmolality is entirely automated. The limiting condition governing whether osmolality can be determined with a vapor pressure osmometer is sample size: samples must be at least 8 μL (Velotta et al. 2014). In our experience, subjects must be at least 2.5 g wet weight to yield enough plasma for a single individual measurement; it may therefore be necessary to pool plasma from multiple small fish.

An alternative technique for determining plasma osmolality measures freezing or melting points, which are reduced by solutes. Freezing point osmometers are widely used in clinical, biopharmaceutical, and multiple manufacturing settings and can handle samples of comparable

size to vapor pressure osmometers. Devices referred to as nanoliter osmometers are also available for handling very small samples (Braslavsky and Drori 2013). Nanoliter-sized droplets of sample are injected with microcapillary tubes into small volumes of oil to prevent evaporation. The samples are mounted on a plate that is cooled below the samples' freezing point, and then the plate is warmed in a controlled fashion. The temperature at which the state transition of each sample is complete is identified by the operator watching the samples with a microscope. From the perspective of a fish biologist, this technique imposes no practical lower limit on sample size, as nanoliter osmometers have been used to measure the osmolality of individual fish larvae (e.g. Sucré et al. 2012). In our experience, determinations can be made on whole blood as well as plasma. Freedom from the constraint of sample volume comes at the expense of a steeper learning curve, more expensive equipment, and longer sample processing time.

Measurement of cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) and anions (Cl-, PO_4^{3-}, SO_4^{2-}) often accompany analysis of plasma osmolality. Given that concentration gradients, passive forces, and mechanisms of active ion transport vary for each cation and anion, measuring each of them can provide unique information; this is especially important in FW where ion concentrations vary dramatically with local conditions. Cation concentrations in fish plasma are often measured via flame photometry (e.g., Bystriansky et al. 2006; Allen and Cech 2007), flame atomic absorption spectrophotometry (FAAS; e.g., Perry and Rivero-Lopez 2012), or inductively coupled plasma atomic emission spectroscopy (ICP-AES). In flame photometry and ICP-AES, the emissions of multiple cations can be measured simultaneously. Absorption measured in FAAS is induced by a beam of a specific wavelength, permitting analysis of one cation at a time. Technical considerations associated with these methods and their use with biological samples are covered in references on biomedical applications in analytic chemistry (e.g., Taylor 1999). Electrolyte analyzers used in medical diagnostics employ ion-specific electrodes to measure concentrations in solution and can be configured to measure cations and anions (Penny and Kieffer 2014, also see section 9.2.4). Finally, colorimetric methods can be used to measure plasma ion levels. For instance, a classic method for measuring chloride in water samples (Zall et al. 1956) is often modified for microplate readers (e.g., Perry and Rivero-Lopez 2012).

9.2.4 Acid-Base Status

Acid-base status of the blood is assessed by measuring pH and PCO_2 of whole blood (not of plasma alone as in ion levels). It is important also to know hemoglobin levels, which are critical determinants of blood buffering capacity (Figure 9.4). Blood pH should be measured at the temperature to which the fish were acclimated and can most easily be measured by glass electrodes (see Heisler 1989) or fiber optic pH sensors (Rummer et al. 2011). There is a trend toward using portable clinical analyzers, such as the i-STAT system, which can be useful under some conditions (but see cautions below). Blood PCO_2 is difficult to measure directly in fish because control values are so low and close to the detection limits of conventional blood gas analyzers. Consequently, it is usually calculated from measurements of total CO_2 either using a Cameron chamber (Cameron 1971) or a Corning CO_2 analyzer. Blood PCO_2 can then be calculated using a rearrangement of the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{pK}' + \log_{10}\left(\frac{[\mathrm{HCO}_3^-]}{\alpha_{\mathrm{CO}_2} \times \mathrm{PCO}_2}\right); \tag{9.1}$$

constants α_{CO2}, the solubility of CO_2 in plasma, and pK′, the apparent pK of the dissociation of carbonic acid, are provided in Boutilier et al. (1984).

Portable clinical analyzers are becoming increasingly available and report a range of parameters important for assessing osmoregulatory and acid-base status. These systems measure only a few parameters directly; other values are calculated from algorithms developed for human blood. Use of clinical analyzers for fish studies requires validation, which has rarely been done. In two studies that have evaluated the use of i-STAT systems in a teleost (Rainbow Trout; Harter et al. 2014) and a shark (Sandbar Shark *Carcharhinus plumbeus*; Harter et al. 2015), findings were mixed. In both studies the i-STAT values reported for blood pH with appropriate correction factors were accurate, but those reported for blood gases (PO_2, hemoglobin oxygen saturation, and PCO_2) were not. Reported values for PCO_2 were either below detection or highly variable in both studies. In contrast, the i-STAT provided values for blood PCO_2 of tropical air-breathing fish were accurate (Damsgaard et al. 2015), presumably because temperature and blood gas conditions in tropical air-breathing fishes are closer to human values for which the i-STAT system was originally designed. In Rainbow Trout (Harter et al. 2014), plasma [Na^+] was also measured by the i-STAT system, but the values reported differed dramatically from those measured by flame photometry, so these systems may be of questionable value for assessing osmoregulatory status. Thus, although clinical analyzers are convenient, the choice to use them should be made carefully and the values reported from studies that use them should be interpreted critically.

9.2.5 Hormones and Receptors

Precautions must be taken with sample collection for many hormones, especially those involved in the stress axis. Bleeding within 5 min of first tank disturbance appears to be necessary to avoid sampling effects on plasma cortisol. In other cases, cannulation may be the only way to avoid stress-associated sampling artifacts (Summerfelt and Smith 1990). Workup can otherwise follow a standard blood sample processing routine of centrifugation within 1 h of collection followed by plasma separation and freezing at –80°C. Because peptide and protein hormones of fishes have only partial sequence similarity, species-specific antibodies may be needed in immunoassays, which are the most common approach for measuring hormone levels. Even when the hormone has the same structure (e.g., cortisol and thyroid hormones) and commercial kits are available, validation procedures (such as parallelism, spiking with known amounts of hormone, induction with releasing hormones) for each new species should be conducted because of interspecific variations in hormone levels and interfering substances in plasma.

Measurement of receptor levels can provide insight into which tissues are likely to be responsive to hormones and whether hormone sensitivity may change during ontogeny or in response to environmental factors such as salinity. Membrane receptors can be quantified with western blots or with “classical” binding studies using radioactively-labeled hormone. Their cellular and intracellular localization of receptors can be detected with immuno- and radiohistochemistry. More details of our current understanding of hormone receptors and greater detail of their measurement can be found in Jeffrey et al. (2022, Chapter 14, this volume).

Further insight into the role of hormones in osmoregulation and acid-base balance can be provided by manipulating hormone levels. Removal of the source of hormones by surgical removal of the endocrine gland (termed hypophysectomy in the case of the pituitary gland)

followed by injection of the hormone of interest is a classical approach (Nishioka 1980). More recently, knockdown and knockout studies have been used (Hwang et al. 2011), and the CRISPR (clustered regularly interspersed short palindromic repeats)-Cas9 system offers even more opportunity for hormone intervention, both positive and negative. Injection of hormones can also be used effectively in intact animals. Use of the homologous (native) hormone is always preferable, since the specificity of heterologous (nonnative) hormones is questionable. The choice of dose and vehicle (solvent) for hormone delivery is important for achieving physiologically relevant levels and timing of release. Single injection of hormones dissolved in saline may be appropriate for rapid acting (minutes to hours) peptides, but steroid, thyroid, and protein hormones with longer-term actions may require more sustained release formulations. These can be achieved by suspension of steroid and protein hormones (e.g., growth hormone) in 1:1 vegetable oil:shortening (Specker et al. 1994), creation of slow release emulsions, silastic or cholesterol capsules, or use of osmotic minipumps (McCormick et al. 1992; see Jeffrey et al. 2022, Chapter 14, for more details on these approaches). Treatment with inhibitors of hormone production or compounds that interfere with the binding of hormones to their receptors can also be effective in delineating hormone function. Some caution should be exercised in interpreting results from the use of these compounds, especially those developed for use in mammals, for which their target and nonspecific actions may not be identical to those of fishes. Ex vivo and in vitro systems have also been widely used for examining the direct actions of hormones on osmoregulatory tissue (see section 9.3.9).

9.2.6 Tissue-level and Whole Body Analyses

While the measurement of plasma ions and osmolality provides the most sensitive indication of critical osmoregulatory responses, measures of tissues other than blood can offer valuable additional information. For example, exposure to hypersaline water can cause whole body weight loss and reduced water content of tissue (Sardella et al. 2009), and there is a general correlation between muscle water content and osmoregulatory capacity (Gutierre et al. 2014). Water content of tissue (termed moisture content), usually in muscle, can be measured by dehydrating 0.2 to 1.0 g of tissue at 60°C to a constant weight, which generally takes 24 to 48 h. Using methods outlined in section 9.2.3, ion content of whole animals, usually larvae or small juveniles, can be quantified after nitric acid digestion and can then be expressed per dry weight of tissue. This approach should be used with some caution. The large "reservoir" of ions in tissues will change more slowly than ions in the blood. To our knowledge, a comprehensive temporal comparison of ion levels in blood and tissue following salinity change has not been undertaken.

Approaches to quantitation of osmolytes in tissues as well as plasma are expanding rapidly. More conventional techniques focus on individual osmolytes. Trimethylamine N-oxide and urea can be quantified using standard spectrophotometry (e.g., Deck et al. 2016). Inositol monophosphate 1 has been quantified in western blots (Kalujnaia et al. 2010). Chromatography is used to profile multiple osmolytes in tissue extracts or plasma; high pressure liquid chromatography (HPLC) can be followed by quantification of peaks by universal refractive index detectors (e.g., Divino et al. 2016). Quantification of osmolytes is increasingly conducted in metabolomics analyses by means of nuclear magnetic resonance spectroscopy (e.g., Jiang et al. 2019) or HPLC coupled with mass spectrometry (Benskin et al. 2014). In all of these analytical techniques, it is critical to evaluate extraction efficiency and analytical accuracy thoroughly with spiking and recovery experiments.

Methods have been developed to measure the intracellular pH (pHi) of different tissues. One involves injecting DMO (5,5-dimethyl-oxaxolidinedione), a weak acid, via catheter into the blood. The nondissociated form diffuses freely across membranes while the dissociated form distributes inversely with $[H^+]$. Tissue pHi is then calculated from the intra- versus extracellular concentration of DMO (see Heisler 1989 for more detail). A much simpler method has recently been developed (metabolic inhibitor tissue homogenate method; Pörtner et al. 1990). Briefly, tissues are rapidly removed following euthanasia, frozen in liquid nitrogen, and pulverized. A chilled metabolic inhibitor solution is added to the sample; the pH of the thawed supernatant represents the mean pHi of the tissue. This technique has been validated for fishes exposed to high water PCO_2 levels (Baker et al. 2009).

9.2.7 Transporters, Channels, and Junction Proteins

Enzyme assays can be used to measure the response of primary active transport proteins to changes in environmental salinity. Most often enzyme activity is estimated by comparing reaction rates between tissue homogenates that contain or lack specific inhibitors, and usually a product of ATP hydrolysis is measured. Endpoint assays measure product after a specified period of time (e.g., Zaugg 1982), whereas kinetic assays continuously monitor the rate of appearance of the product (e.g., McCormick 1993a). Endpoint assays are often simpler but are more subject to error from variations in reaction start time or inhibition. Kinetic assays also permit determination of the linear rate of activity over a wide dynamic range. Temperature must be tightly controlled (±0.5°C) in all assays. Validating each assay, which entails optimizing concentrations of reaction substrates, the ions activating the reaction, and inhibitors, should be done for each species. Gill NKA has been the most widely assayed transport protein in fish because of the central importance and abundance of the transporter and the relative ease with which its activity can be measured. Conditions for NKA assays have been optimized for a number of fish species and the inhibitor used, ouabain, is highly specific. Activity of H^+-ATPase is now being measured using bafilomycin as an inhibitor, although to our knowledge optimal assay conditions (inhibitor, pH, and ion concentrations) for fish have not been published to date. N-ethymaleimide (NEM) is less preferable as an inhibitor of H^+-ATPase because NEM is specific only at low concentrations (10 μM; Forgac 1989), much lower than has been used in most fish studies. Activity of Ca^{2+}-ATPase has also been measured in gill and gut of several fish species (Flik et al. 1984), but the lack of a specific inhibitor limits interpretability of results. Tissue for enzyme assays should be snap frozen on dry ice or liquid nitrogen, stored at –80°C and measured within six months. A single freeze–thaw event will destroy all transport enzyme activity.

The activities of primary active ion transport proteins are usually interpreted as indicators of their abundance in the tissue of interest. However, the activity that is measured in an enzyme assay may also reflect other properties of the enzyme and its environment. These include the proportion of the protein that is membrane bound, its degree of phosphorylation, its binding to other molecules (such as the NKA-activating protein FYXD), and membrane properties. For most teleost species, gill NKA activity is higher in SW than in FW, and some species exhibit lower activity at isosmotic salinity, resulting in a U- or J-shaped relationship between salinity and gill NKA activity (McCormick 1995). However, this is not universal, and some euryhaline fishes (e.g., flounder) of marine origin have higher gill NKA activity in FW than in SW (Sampaio and Bianchini 2002). In addition to measuring abundance, enzyme activity can also be used to examine differences in ion affinity and activation of ion transport

enzymes that may be altered by environmental salinity. These differences are likely due to expression of different isoforms (paralogs) of ion transport enzymes such as NKA, an area of active research in the field (Richards et al. 2003; McCormick et al. 2013; Dalziel et al. 2014).

Abundances of ion transport and junction proteins can be measured using specific antibodies. This is most often applied through the use of semiquantitative western blots or more quantitative enzyme immunoassays. Antibodies can also be used in immunohistochemical approaches to localize transport and junction proteins to specific cell types and locations within or between cells, as detailed in the next section on ionocytes. In western blots, tissues are homogenized and then reduced using Lammeli's buffer. Constituents are then separated by size and charge on an electrophoretic gel. The proteins are then transferred to a membrane that is first exposed to the transporter-specific antibody and then to a secondary antibody bound to a signaling molecule, such as fluorescence or a colorimetric enzyme. Basic protocols for western blots can be found in Kurien and Scofield (2006), and examples of their application in fish osmoregulatory studies can be found in McCormick et al. (2013). Enzyme immunoassays are often more sensitive and quantitative, as well as faster, than western blots but can be more difficult to set up because they require purified ligand (the full-length protein or peptide used to create the antibody) and one or two antibodies that are highly specific to the protein. Antibody specificity is critical to the success of these techniques. Homologous antibodies (those made to the known amino acid sequence of the target species) are preferable to heterologous (cross-species) antibodies. Nonetheless, heterologous antibodies have been widely used in fish because there is often sufficient sequence similarity in conserved regions of the protein that are used for generating antibodies. For example, the α5 and T4 antibodies (both available from the Developmental Studies Hybridoma Bank, http://dshb.biology.uiowa.edu/) have been widely used for detecting NKA and NKCC, respectively, in a wide variety of fish species. It should be noted that in some species the T4 antibody recognizes both NKCC and NCC (Hiroi and McCormick 2012) and that NCC-specific antibodies have been developed. When using these approaches on a previously unexamined species, antibodies and their western blots or immunoassay procedures should be validated, which includes verification of a single product of the appropriate molecular weight and parallelism of measurement (e.g., doubling of signal with doubling of protein loading; McDonough et al. 2015). For homologous antibodies the peptide used to generate the antibody can also be used to displace the signal, providing further verification of the antibodies' specificity.

Quantitative or real-time PCR is increasingly employed to measure messenger RNA (mRNA) levels of ion transporters as more genomes of nonmodel species become available. Protocols are widely available, but measurements require some level of training and can be relatively expensive. Whole-transcriptome analysis (e.g., Kozak et al. 2014; Velotta et al. 2017) will play a larger role in osmoregulatory studies as costs continue to decrease. It is important to recognize that several post-transcriptional processes modulate the variability in gene expression, so that the abundance of mRNA may not accurately reflect the abundance of the transport protein. Nonetheless, molecular genetic approaches can provide important initial indication of genes that may be involved in responding to changing environmental ion levels.

9.2.8 Ionocytes

The identification and enumeration of ionocytes can be useful for determining the organismic response to salinity and the role of ionocytes in ion regulation. In living tissue (ex vivo approaches), ionocytes can be identified using fluorescent mitochondria markers (Hiroi et al.

1999) or a fluorescent derivative of ouabain (McCormick 1990). Immunohistochemical (IHC) approaches using antibodies to specific ion transporters, especially NKA, are now widely used to identify ionocytes. After fixation, samples are embedded in paraffin or are frozen for sectioning, and small samples can be examined using whole mount (nonsectioned) IHC preparations, often using laser confocal microscopy. Freeze-sectioning can permit better antibody binding but at the cost of reduced cellular details. Standard protocols for IHC techniques can be found in Osborn and Isenberg (1998) and application to fish found in Christensen et al. (2012) and Wilson et al. (2000). Immunohistochemical approaches are useful for localizing transporters within ionocytes and determining the abundance and distribution of different ionocyte types. Freshwater ionocytes have been distinguished from SW ionocytes with use of antibodies to salinity-specific isoforms of NKA in salmonids and antibodies to other ion-absorbing or ion-secreting transporters in other fishes (Hiroi and McCormick 2012). Diversity within FW ionocytes has been revealed with antibodies to various transporters involved in ion uptake (Hwang et al. 2011). Scanning electron microscopy (SEM) can often distinguish FW and SW ionocytes based on their differential apical morphologies. This approach measures ionocytes that are exposed to the environment, which are likely active ionocytes since inactive ionocytes are presumed to be covered by gill pavement cells. An innovative combination of fluorescent approaches combined with SEM can measure both active and inactive ionocytes (Choi et al. 2011). Regardless of the approach used, efforts to assess the abundance of ionocytes must consider how they are distributed across the complex three-dimensional surface of the gill (Christensen et al. 2012). Many of these approaches can also be applied to the gut and kidney, which have not been as widely examined as the gill.

9.2.9 Ion Fluxes, Electrophysiology, and in Vitro Approaches

While measurement of plasma ion levels and osmolality is important in assessing ionoregulatory status in fish, changes often take a long time to manifest. A more immediate measure of impact is obtained by looking at branchial ion fluxes. This can be as simple as placing a fish in a chamber and measuring the net change in the ion of interest over time, where appearance of an ion in the water indicates net loss from the fish and disappearance of an ion from the water indicates net gain by the fish (see Wood 1992). The basis for these net changes in FW can be investigated if a radioisotope exists for the ion of interest, such as ^{22}Na: the radioisotope is added to the water in which a fish resides and then the disappearance of the radioisotope from the water, or appearance in the fish, is measured over a fixed time. This provides the unidirectional uptake of the respective ion, which when combined with a measurement of net flux can be used to calculate unidirectional efflux. These parameters together give a comprehensive picture of how a change in environment or developmental stage affects both active and passive ion fluxes (Wood 1992). These approaches have been especially useful for understanding the impacts of metals on osmoregulation (Box 9.3; Figure 9.6).

Ussing chambers, which use a flat membrane such as skin or intestine to separate two chambers, have been used to characterize ion fluxes and electrophysiological characteristics of tissues involved in ion transport. The opercular ("inner cheek") membrane of some teleost species contains ionocytes that are nearly identical to those of the gill, and Ussing chamber studies on this tissue have been widely used as a model to determine the mechanisms involved in ion uptake and secretion by the gill (Zadunaisky 1984). More recently, Ussing chambers have been used with fish intestine to examine ion and water transport, electrophysiologi-

Box 9.3 Case Study: Metal Toxicity

Understanding the effects of metal toxicity is another area of recent progress. While initial studies indicated that many metals exert their toxic effects on the gill through cell swelling, mucous production, and suffocation, recent work has shown that toxic effects arise at lower concentrations through disruption of ion regulation (Wood 2012). For example, both silver and copper compete with Na^+ for apical uptake and inhibit basolateral NKA activity. Consequently, in FW, active Na^+ and Cl^- uptake are inhibited, plasma levels of these ions and osmolality fall, and acid-base balance is compromised. Osmoregulatory impairment leads to fluid shifts and the release of catecholamines and cortisol, which together set up a cascade elevating blood viscosity and blood pressure, ultimately leading to cardiovascular collapse and death (Figure 9.6). Insights into the osmoregulatory mechanisms of impairment have promoted the development of a Biotic Ligand Model that predicts the toxicity of different metals under various environmental conditions; this model has been widely adopted by regulators responsible for generating water quality criteria (Paquin et al. 2012).

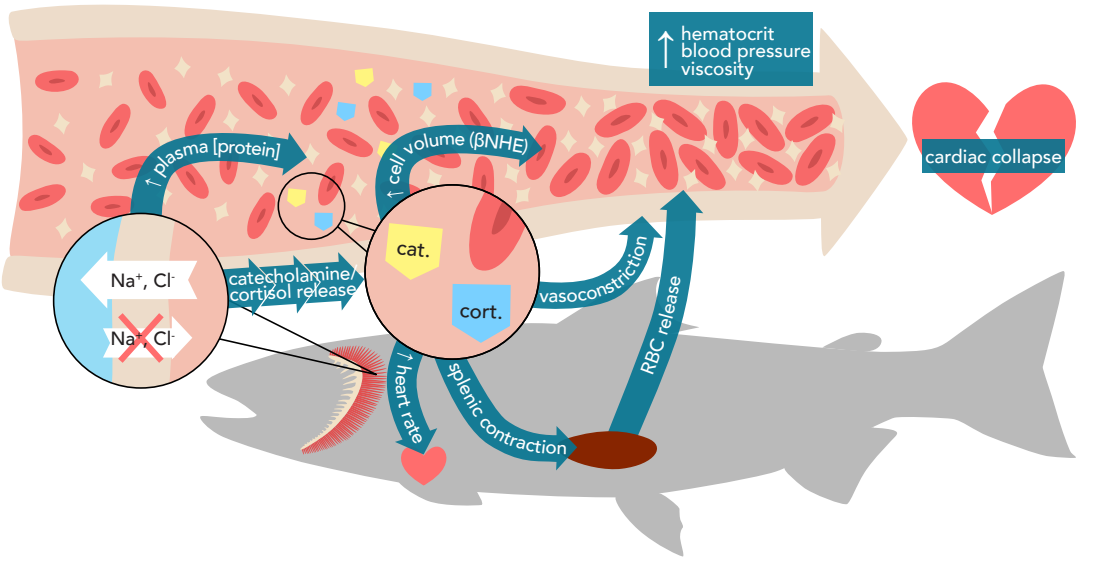

Figure 9.6. Proposed sequence of events through which ionoregulatory disruption in freshwater fish exerts its lethal effects. Branchial ion loss and/or impaired uptake (as occurs in low pH water or during exposure to some metals) leads to a reduction in plasma ion levels and osmolality, which results in fluid volume disturbances that reduce blood volume and increase plasma protein concentration. The stress-induced release of catecholamines (cat.) and cortisol (cort.) results in an elevation in heart rate and general vasoconstriction, as well as contraction of the spleen and red blood cell (RBC) release. Catecholamines also activate beta adrenergic Na^+/H^+ exchangers (βNHE) on the RBC membrane that result in red cell swelling. Combined, all these changes result in an elevation in hematocrit, blood viscosity, and blood pressure, which result in cardiovascular collapse and death. Modified from Milligan and Wood (1982). Figure created by Jacelyn Shu.

cal characteristics, and their regulation. Methods for use of Ussing chambers for opercular membranes and intestine can be found in McCormick (1993b) and Carvalho et al. (2012), respectively. Ion-sensitive vibrating probes, which can measure differences in current and ion concentrations at the surface of cells, were used to identify ionocytes as the site of Cl^- secretion in SW fish (Foskett and Scheffey 1982) and have subsequently been used to examine both the mechanisms and regulation of ion uptake and secretion by ionocytes (Hwang et al. 2011). Ion- and pH-sensitive dyes can also be used to examine mechanisms of ion transport in larvae (where ionocytes are located on the yolk sac; Hwang et al. 2011) and gill explants (Lee et al. 2016).

A variety of moderate- to long-term in vitro preparations have been developed that have utility for measuring ion transport and its regulation. Gill and opercular membrane explant systems have been used to examine the hormonal control of ion transport and junction proteins and ionocytes (McCormick and Bern 1989; McCormick 1993b; Breves et al. 2013). An isolated "yolk-ball" system consisting of the larval yolk sac and yolk (without the rest of the larvae) has also been utilized to examine ionocyte function and regulation (Shiraishi et al. 2001). These explant systems last for only several days, and during that time ionocytes will gradually be lost because of cell turnover. Long-term primary cell polarized cultures that contain primarily pavement cells have been useful for examining the development and regulation of membrane properties, transepithelial potential, and junction proteins (Schnell et al. 2016). To date a long-term cell culture system containing mature ionocytes that would allow examination of active ion transport has not been developed.

9.2.10 Behavioral Methods and Drinking Rates

A whole-organism process that has received relatively little study is behavioral osmoregulation. Some fishes live in habitats that are fine grained with respect to salinity, such as estuaries and tidal creeks, and could exhibit salinity preferences as a means of maintaining osmoregulatory homeostasis. The potential for measuring behavioral salinity preference was first experimentally tested by Shelford and Powers (1915) and Wells (1915), who set up tanks that offered a choice of salinity and recorded where subjects moved in the tank. The salinity difference was maintained by continuous flow of water of contrasting salinity at opposite ends of the tank. Salinity choice tanks commonly use partitions that partially separate two or more chambers of contrasting salinities; the partitions are overtopped by water providing an "aqueous bridge" that subjects can traverse (e.g., Lerner at al. 2007; Barrett et al. 2009). Movements of subjects between different salinity levels can detected with a tag reader or camera; one study even employed an apparatus that changed the salinity of tanks according to the choices of the subject (Serrano et al. 2010), ostensibly to permit more precise estimates of salinity preference. Salinity gradient tanks have multiple partitions separating volumes with a range of salinity levels. An original design constructed with partitions that separate water only in the deeper portions of the tank (Staaland 1969) was improved by adding intervening partitions that separate water in the shallower portions (Fivizzani and Spieler 1978), yielding a more persistent gradient. Studies using this design include Lankford and Targett (1994) and McManus et al. (2014). In our experience, setting up the gradient is facilitated by outfitting the tank walls with slots that enable additional partitions to be placed on top of the lower partitions for filling the tank, which then can be removed for testing. Other tank design considerations include the number of partitions (often, three or four lower partitions are used) and

tank size (so that subjects are able and willing to explore the entire gradient). An important experimental design consideration is the need for control treatments, comprising tanks with no salinity gradient, to quantify the extent to which subject position is shaped by edge effects, such as the tendency to stay at one end of the tank. Several investigators have suggested cylindrical gradient tanks to obviate edge effects (Kölsch et al. 2010; He and Xie 2013). Another set of approaches gives subjects a choice between flows of contrasting salinities, which is an especially reasonable design when working with species that naturally orient into flowing water (e.g., galaxiids [Hale et al. 2008] and eels [Edeline et al. 2006]). Here again, choice between salinity levels should be analyzed with reference to control conditions in which both flows offer the same salinity level.

Osmoregulating ray-finned fishes drink to balance diffusive losses in SW. The drinking rate of smaller fish is quantified by measuring the uptake of labeled compounds added to the water. Most commonly, radiolabeled tracers, such as tritiated polyethylene glycol (e.g., Sackville et al. 2012), ^{51}Cr-EDTA (e.g., Varsamos et al. 2004; Schwarz and Allen 2014), ^{14}Cdextran (Eddy 2007) are used. The gut is ligated, dissected, and digested for scintillation counting; smaller fish can be digested whole. The potential for the digestion agent to quench radioactivity should be checked (Schwarz and Allen 2014). Blood and organ sampling are conducted to confirm that tracers are not absorbed into the bloodstream. Radiotracer counts have been taken from intact anesthetized fish (45–85 g river lamprey; Rankin et al. 2001). Uptake is quantified as ratio of counts in gut versus the medium, divided by time for a drinking rate. As an alternative to radioisotope labeling, Yaacob et al. (2016) measured uptake of a fluorescent material (fluorescein isothiocyanate-labeled dextran) in larvae, which were measured intact in a microplate reader. Uptake was related to a standard dilution curve. Phenol red has been used in Antarctic fishes (Petzel 2005), which have no blood pigments so that the retention in gut tissue can be readily confirmed, but this approach should be applicable to other species as well. In larger fish such as eels (Takei et al. 1998) or sharks (Anderson et al. 2002), drinking has been measured more directly: water was measured from an esophageal catheter and was replaced by another catheter in the stomach.

9.3 DATA AND OUTCOMES

9.3.1 Experimental Design

Well-designed experiments test a hypothesis about the effect of one or more controlled conditions and account for, or minimize the effect of, various other influences on the response of interest. The response of fish to salinity change can best be assessed in reference to a control group that remains in the unaltered salinity and when the treatment and control groups are measured at the same time points. It is important to include replicate tanks at each salinity because random effects arising from uncontrolled conditions in any single tank can influence responses (see section 9.4.2), especially over long-term confinements. Care should be taken to monitor and match controlled conditions. Temperatures should be either kept constant or matched for each of the salinity groups, and if subjects are fed, ration should be uniform across treatments. Water quality variables that are affected by density and feeding rate, such as pH and ammonia, should be carefully monitored.

The time course over which sampling should occur will depend on the specific hypothesis and may be guided by previous similar studies. In general, changes in plasma ions and mortality rates are greatest in the first 1 to 3 d after a large change in salinity; sampling frequently over this time period is necessary to characterize acute responses. Similarly, high frequency sampling can be important in profiling mRNA responses underlying osmoregulation (Whitehead et al. 2011). On the other hand, some species appear to be "resistant" to salinity change, being tolerant in the short term but unable to osmoregulate in the long term (Hiroi and McCormick 2007). Thus, sampling over short-term (several days) and long-term (weeks) time courses will uncover both acute and acclimation phase responses to salinity change (Figure 9.3). When sampling occurs daily it is best done at the same time to minimize the influence of diel variations in physiological parameters, especially if hormones are an endpoint.

9.3.2 Data Analysis

Analysts of osmoregulatory physiology use a wide array of experimental designs and could therefore apply many different methods for data analysis. Nonetheless, two statistical approaches can (or should) be frequently applied in osmoregulation experiments; both are readily executed in software such as SAS or R.

One approach deserving more widespread use is mixed linear models because they properly account for variance in experimental designs that manipulate subjects in groups, such as those sharing a tank. In such designs there is a potential problem of **pseudoreplication** (Hurlbert 1984). Measured responses of individuals from a single tank are not independent replicate responses to a fixed treatment such as a salinity manipulation; their responses will be more similar to each other than to those of individuals from a different tank receiving the same fixed treatment because of uncontrolled random differences among tanks. Mixed linear models are generalizations of linear models (such as analysis of variance) that take into account correlations among subjects resulting from these random effects. The investigator is not interested in precisely how each tank affected the response; instead, each tank is regarded as a random sample from an imaginary set of tanks, and the investigator needs to account for that random variability so as to properly determine the influence of the fixed salinity effect. Note that in such designs, true replication occurs at the tank level, and it is essential that the same treatment level, such as a salinity, be imposed on multiple tanks.

Another approach that readers of this chapter should be familiar with is a general class of models referred to as survival analysis, which are designed to deal with data on the timing of an event within a group of subjects, such as mortality in a salinity challenge experiment. Rather than restricting attention on the LC50 (discussed in section 9.2.1), more use is made of data by modeling the timing of mortality for each group. An important benefit of employing survival analysis is the capability of dealing with "censoring" of subjects for which the data on survival is incomplete. For example, a salinity challenge experiment might withdraw some subjects from the trial, perhaps to sample gill tissue. Until they are taken out of the trial, their survival is meaningful (they are "at risk" in the parlance), but their removal means that their ability to survive the experimental conditions cannot be measured: these subjects are "right-censored." Survival models appropriately include such subjects in the estimates of survival probability up to the point of censoring. Investigators may choose parametric or nonparametric models for survival analysis, differing with respect to whether survival times are assumed to follow theoretical distributions. Perhaps most widely used in salinity chal-

lenge experiments is the Cox proportional hazards regression model, which enables analysts to estimate how potentially important covariates (e.g., subject size; Velotta et al. 2014) affect mortality while making no assumptions about the distribution of underlying mortality risk. One accessible book on the subject of survival analysis is Lee (1980).

9.4 EMERGING IDEAS/FUTURE DIRECTIONS

The field of comparative osmoregulatory physiology has already seen the regular incorporation of molecular genetic approaches such as quantitative PCR and gene knockdown for determining genes and their proteins that are involved in ion, water, and acid-base balance. Use of CRISPR and other as-yet undiscovered genetic manipulations will make it increasingly easy to identify the ion and acid-base regulatory function of specific genes in nonmodel species. The integration between physiology and genetics has already provided great insight into the evolutionary basis of osmoregulatory physiology. Classic quantitative genetic approaches have identified specific genes that are under selection, and this is an area in which great advances will be made as techniques improve. Comparison of transcriptional responses of fish populations with different osmoregulatory capacity have uncovered genes whose regulation appears to be critical for variations in salinity tolerance (Whitehead et al. 2011; Velotta et al. 2017). As costs of transcriptome sequencing decrease there will be increasing opportunities to examine whole transcriptome responses to environmental salinity, with opportunities to see whether different clades of fish have used common or unique evolutionary pathways to achieve euryhalinity. Proteomic and metabolomic approaches will move us toward an even more physiologically relevant understanding of global cellular responses. Advances in real-time imaging of specific proteins and their activation within cells has the opportunity to vastly improve our understanding of osmoregulation and acid-base balance of fishes at the cellular and protein level. The extremely high density of ion transport proteins and mitochondria within ionocytes would make them a powerful model system for analysis of membrane-protein interactions, rapid protein activation, and oxidative stress (Hiroi and McCormick 2012).

The role of behavior in dealing with osmoregulatory challenges has received relatively little attention. Of course, the first response of any animal placed in an unfavorable environment is to leave if that is an option. There are a range of behavioral assays being developed in fish to identify whether fish "choose" environments that may be physiologically optimal and the degree to which they can sense and avoid changing environments that are less desirable or unfavorable. For example, in some euryhaline fish, isosmotic environments may reduce the cost associated with osmoregulation and may seem physiologically optimal, but do fish choose these salinities if given the option? Designing experiments that incorporate both choice and physiological responses will shed light on these important questions. The application of physiological measurements to field studies also has great potential. Technological advances such as real-time telemetry that can simultaneously monitor animal movement in space and time, as well as conditions of both the external (salinity, temperature, oxygen) and internal environment (ions, pH, hormones), will rapidly advance our understanding of how fish respond to challenges in their natural environment.

Habitat loss, contaminants, and climate change due to human activity have resulted in unprecedented levels of extinctions and reduced populations. The incorporation of physiological and endocrine indicators for conservation purposes is the basis for the developing fields of "conservation physiology" and "conservation endocrinology" (Wikelski and Cooke

2006; McCormick and Romero 2017). Lethal and sublethal indicators can be used to estimate environmental thresholds and how individuals and even populations may respond to different conditions (Brauner and Richards 2020). As discussed above, disruption of ionoregulation through acid rain or metal exposure in FW fish leads to a reduction in plasma ion levels (of 20–30%) that, if uncorrected, results in internal fluid shifts that ultimately lead to cardiovascular collapse and death (Figure 9.6). The identification of unifying mechanisms of toxicity or sublethal impact is central to establishing thresholds that can be incorporated into models to make predictions about changing environments (McGeer et al. 2000; Brauner and Wood 2002). Understanding underlying mechanisms will be an important unifying framework for other environmental challenges related to climate change and habitat destruction that have the potential to affect osmoregulation and acid-base balance in fish. Dams have impacted the migratory routes on **diadromous** species, and their direct and indirect impacts on physiology and survival during transitions between FW and SW are only partly understood. Studies to identify specific physiological mechanisms used by fish to respond to changing environments often employ a single variable (e.g., salinity) to reduce unwanted variation. However, in the natural world, many parameters change simultaneously both inside and outside the animal. It will be important to understand the interaction of salinity change with other environmental variable such as temperature, pH, and food to understand more fully how environmental change influences survival and growth of different species in a changing world.

ACKNOWLEDGMENTS

We thank Dan Hall, Diogo Ferreira-Martins, and Jacelyn Shu for help in preparing figures. A draft of this chapter was improved through the comments of Jessica Norstog, Chris Glover, Silvia Gutierre, and Caleb Hasler. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

9.5 TERMS

Amphidromous: diadromous fishes that reproduce in freshwater, pass to the sea as newly hatched larvae where they feed and grow, then return to freshwater as juveniles for another period of feeding and growth, followed by reproduction

Amphihaline: capable of surviving in freshwater and seawater

Anadromous: diadromous fishes that spend part of their lives in the sea and migrate to freshwater to breed and for early development

Catadromous: diadromous fishes that spend part of their lives in freshwater and migrate to the sea to breed and for early development

Diadromous: migratory fishes that move between the sea and freshwater

Euryhaline: capable of surviving in a wide range of salinity

Halohabitat: ionic composition of a habitat

Halotolerance: capacity for survival in water with different ion levels

Hypoxemia: low internal (blood) levels of oxygen

Hypoxia: low environmental (water) levels of oxygen

Ionocyte: specialized cell in the gill and skin that transports ions to maintain internal (blood) homeostasis; also known as "chloride cells" and "mitochondrion-rich cells"

Pseudoreplication: inflation in the number of replicates arising from failure to account properly for how subjects are distributed in experimental units receiving fixed treatments, such as tanks in which salinity has been manipulated

Stenohaline: capable of surviving in only a narrow range of salinity

9.6 REFERENCES

Allen, P. J., and J. J. Cech, Jr. 2007. Age/size effects on juvenile green sturgeon, *Acipenser medirostris*, oxygen consumption, growth, and osmoregulation in saline environments. Environmental Biology of Fishes 79:211–229.

Anderson, W. G., Y. Takei, and N. Hazon. 2002. Osmotic and volaemic effects on drinking rate in elasmobranch fish. Journal of Experimental Biology 205:1115–1122.

Baker, D. W., C. May, and C. J. Brauner. 2009. A validation of intracellular pH measurements in fish exposed to hypercarbia: the effect of duration of tissue storage and efficacy of the metabolic inhibitor tissue homogenate method. Journal of Fish Biology 75:268–275.

Baker, D. W., B. Sardella, J. L. Rummer, M. Sackville, and C. J. Brauner. 2015. Hagfish: champions of CO_2 tolerance question the origins of vertebrate gill function. Scientific Reports 5:11182.

Ballantyne, J. S., and J. W. Robinson. 2010. Freshwater elasmobranchs: a review of their physiology and biochemistry. Journal of Comparative Physiology+ B: Biochemical, Systemic, and Environmental Physiology 180:475–493.

Barrett, R. D. H., T. H. Vines, J. S. Bystriansky, and P. M. Schulte. 2009. Should I stay or should I go? The ectodysplasin locus is associated with behavioural differences in Threespine Stickleback. Biology Letters 5:788–791.

Behrens, J. W., M. H. van Deurs, and E. A. F. Christensen. 2017. Evaluating dispersal potential of an invasive fish by the use of aerobic scope and osmoregulation capacity. PLoS ONE 12:e0176038.

Benskin, J. P., M. G. Ikonomou, J. Liu, N. Veldhoen, C. Dubetz, C. C. Helbing, and J. R. Cosgrove. 2014. Distinctive metabolite profiles in in-migrating Sockeye Salmon suggest sex-linked endocrine perturbation. Environmental Science and Technology 48:11670–11678.

Betancur-R., R. G. Ortí, and R. A. Pyron. 2015. Fossil-based comparative analyses reveal ancient marine ancestry erased by extinction in ray-finned fishes. Ecology Letters 18:441–450.

Black, M. C. 2000. Collection of body fluids. Pages 513–527 *in* G. K. Ostrander, editor. The laboratory fish. Academic Press, San Diego, California.

Boutilier, R. G., T. A. Heming, and G. K. Iwama. 1984. Appendix: physicochemical parameters for use in fish respiratory physiology. Pages 403–440 *in* W. S. Hoar and D. J. Randall, editors. Fish physiology, volume 10. Gills, Part B: anatomy, gas transfer and acid-base regulation. Academic Press, New York.

Braslavsky, I., and R. Drori. 2013. LabVIEW-operated novel nanoliter osmometer for ice binding protein investigations. Journal of Visualized Experiments 72:e4189.

Brauner, C. J. and J. G. Richards. 2020. Physiological performance in aquaculture: using physiology to help define the optimal conditions for growth and environmental tolerance. Pages 83–121 *in* T. Benfey, A. P. Farrell and C. J. Brauner, editors. Fish physiology, volume 38. Aquaculture. Elsevier, New York.

Brauner, C. J., R. B. Shartau, C. Damsgaard, A. J. Esbaugh, R. W. Wilson, and M. Grosell. 2019. Acid-base physiology and CO_2 homeostasis: regulation and compensation. Pages 69–132 *in* M. Grosell, P. L. Munday, A. P. Farrell, and C. J. Brauner, editors. Fish physiology, volume 37. Carbon dioxide. Elsevier, New York.

Brauner, C. J., and C. M. Wood. 2002. Ionoregulatory development and the effect of chronic silver exposure on growth, survival, and sublethal indicators of toxicity in early life stages of Rainbow Trout (*Oncorhynchus mykiss*). Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 172:153–162.

Breves, J. P., S. B. Serizier, V. Goffin, S. D. McCormick, and R. O. Karlstrom. 2013. Prolactin regulates transcription of the ion uptake Na^+/Cl^- cotransporter (NCC) gene in Zebrafish gill. Molecular and Cellular Endocrinology 369:98–106.

Bringolf, R. B., T. J. Kwak, W. G. Cope, and M. S. Larimore. 2005. Salinity tolerance of flathead catfish: implications for dispersal of introduced populations. Transactions of the American Fisheries Society 134:927–936.

Brown, J. A., J. A. Oliver, I. W. Henderson, and B. A. Jackson. 1980. Angiotensin and single nephron glomerular filtration in the trout *Salmo gairdneri*. American Journal of Physiology—Regulatory Integrative and Comparative Physiology 239:R509–R514.

Bucking, C., and C. M. Wood. 2006. Gastrointestinal processing of Na^+, Cl^-, and K^+ during digestion: implications for homeostatic balance in freshwater Rainbow Trout. American Journal of Physiology—Regulatory Integrative and Comparative Physiology 291:R1764–R1772.

Bystriansky, J. S., J. G. Richards, P. M. Schulte, and J. S. Ballantyne. 2006. Reciprocal expression of gill Na^+/K^+-ATPase alpha-subunit isoforms alpha 1a and alpha 1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. Journal of Experimental Biology 209:1848–1858.

Cameron, J. 1971. A rapid method for determination of total carbon dioxide in small blood samples. Journal of Applied Physiology 31:632–634.

Carvalho, E. S. M., S. F. Gregório, D. M. Power, A. V. M. Canário, and J. Fuentes. 2012. Water absorption and bicarbonate secretion in the intestine of the sea bream are regulated by transmembrane and soluble adenylyl cyclase stimulation. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 182:1069–1080.

Choi, J. H., K. M. Lee, M. Inokuchi, and T. Kaneko. 2011. Morphofunctional modifications in gill mitochondria-rich cells of Mozambique Tilapia transferred from freshwater to 70% seawater, detected by dual observations of whole-mount immunocytochemistry and scanning electron microscopy. Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology 158:132–142.

Christensen, A. K., J. Hiroi, E. T. Schultz, and S. D. McCormick. 2012. Branchial ionocyte organization and ion-transport protein expression in juvenile alewives acclimated to freshwater or seawater. Journal of Experimental Biology 215:642–652.

Clark, T. D., M. R. Donaldson, S. M. Drenner, S. G. Hinch, D. A. Patterson, J. Hills, V. Ives, J. J. Carter, S. J. Cooke, and A. P. Farrell. 2011. The efficacy of field techniques for obtaining and storing blood samples from fishes. Journal of Fish Biology 79:1322–1333.

Clarke, W. C. 1982. Evaluation of the seawater challenge test as an index of marine survival. Aquaculture 28:177–183.

Clarke, W. C., R. L. Saunders, and S. D. McCormick. 1996. Smolt production. Pages 517–567 *in* W. Pennel and B. A. Barton, editors. Principles of salmonid culture, volume 29. Elsevier, Amsterdam.

Cooper, C. A., M. D. Regan, C. J. Brauner, E. S. R. De Bastos, and R. W. Wilson. 2014. Osmoregulaory bicarbonate secretion exploits H^+-sensitive haemoglobins to autoregulate intestinal O_2 delivery in euryhaline teleosts. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 184:865–876.

Currie, S., and S. L. Edwards. 2010. The curious case of the chemical composition of hagfish tissues—50 years on. Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology 157:111–115.

Dalziel, A. C., J. Bittman, M. Mandic, M. Ou, and P. M. Schulte. 2014. Origins and functional diversification of salinity-responsive Na^+, K^+ ATPase alpha1 paralogs in salmonids. Molecular Ecology 23:3483–3503.

Damsgaard, C., L. T. H. Gam, D. D. Tuong, P. V. Thinh, D. T. Huong Thanh, T. Wang, and M. Bayley. 2015. High capacity for extracellular acid–base regulation in the air-breathing fish *Pangasianodon hypophthalmus*. Journal of Experimental Biology 218:1290–1294.

Deck, C. A., A. B. Bockus, B. A. Seibel, and P. J. Walsh. 2016. Effects of short-term hyper- and hypoosmotic exposure on the osmoregulatory strategy of unfed North Pacific Spiny Dogfish (*Squalus suckleyi*). Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 193:29–35.

Divino, J. N., M. Y. Monette, S. D. McCormick, P. H. Yancey, K. G. Flannery, M. A. Bell, J. L. Rollins, F. A. von Hippel, and E. T. Schultz. 2016. Osmoregulatory physiology and rapid evolution of salinity tolerance in recently lake-introduced Threespine Stickleback. Evolutionary Ecology Research 17:179–201.

Eddy, F. B. 2007. Drinking in juvenile Atlantic Salmon (*Salmo salar* L.) in response to feeding and activation of the endogenous renin-angiotensin system. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 148:23–28.

Edeline, E., P. Lambert, C. Rigaud, and P. Elie. 2006. Effects of body condition and water temperature on *Anguilla anguilla* glass eel migratory behavior. Journal of Experimental Marine Biology and Ecology 331:217–225.

Edwards, S. L., and W. S. Marshall. 2013. Principles and patterns of osmoregulation and euryhalinity in fishes. Pages 1–44 *in* S. D. McCormick, C. J. Brauner, and A. P. Farrell, editors. Fish physiology, volume 32. Euryhaline fishes. Academic Press, Amsterdam.

Eliason, E. J., A. Ekström, A. Gräns, G. K. Cox and E. Sandblom. 2022. Cardiovascular system. Pages 309–345 *in* S. Midway, C. Hasler, and P. Chakrabarty, editors. Methods in fish biology, 2nd edition. American Fisheries Society, Bethesda, Maryland.

Evans, D. H., P. M. Piermarini, and K. P. Choe. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiological Reviews 85:97–177.

Faulk, C. K., and G. J. Holt. 2006. Responses of cobia *Rachycentron canadum* larvae to abrupt or gradual changes in salinity. Aquaculture 254:275–283.

Fivizzani, A. J., and R. E. Spieler. 1978. Modified Staaland device with automatic recording techniques for determining salinity preference in fishes. Journal of the Fisheries Research Board of Canada 35:910–912.

Flik, G., S. E. Wendelaar Bonga, and J. C. Fenwick. 1984. Ca^{2+}-dependent phosphatase and Ca^{2+}dependent ATPase activities in plasma membranes of eel gill epithelium. II. Evidence for transport high-affinity Ca^{2+}-ATPase. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology 79:9–16.

Forgac, M. 1989. Structure and function of vacuolar class of ATP-driven proton pumps. Physiological Reviews 69:765–796.

Foskett, J. K., and C. Scheffey. 1982. The chloride cell: definitive identification as the salt-secretory cell in teleosts. Science 215:164–166.

Gilmour, K. M., and S. F. Perry 2018. Conflict and compromise: using reversible remodeling to manage competing physiological demands at the fish gill. Physiology 33:412–422.

Gutierre, S. M. M., J. R. S. Vitule, C. A. Freire, and V. Prodocimo, 2014. Physiological tools to predict invasiveness and spread via estuarine bridges: tolerance of Brazilian native and worldwide introduced freshwater fishes to increased salinity. Marine and Freshwater Research 65:425–436.

Hale, R., B. J. Downes, and S. E. Swearer. 2008. Habitat selection as a source of inter-specific differences in recruitment of two diadromous fish species. Freshwater Biology 53:2145–2157.

Harter, T. S., P. R. Morrison, J. W. Mandelman, J. L. Rummer, A. P. Farrell, R. W. Brill, and C. J. Brauner. 2015. Validation of the i-STAT system for the analysis of blood gases and acid–base status in juvenile Sandbar Shark (*Carcharhinus plumbeus*). Conservation Physiology 3:cov002.

Harter, T. S., R. B. Shartau, C. J. Brauner, and A. P. Farrell. 2014. Validation of the i-STAT system for the analysis of blood parameters in fish. Conservation Physiology 2:cou037.

Hasler, C. T., D. Butman, J. D. Jeffrey, and C. D. Suski. 2016. Freshwater biota and rising pCO_2? Ecology Letters 19:98–108.

He, X., and C. Xie. 2013. A novel development of the Staaland device for testing salinity preference in fish. Marine and Freshwater Behaviour and Physiology 46:21–32.

Heisler, N. 1986. Acid-base regulation in animals. Elsevier, Amsterdam.

Heisler, N. 1989. Parameters and methods in acid-base physiology. Pages 305–332 *in* C. R. Bridges and P. J. Butler, editors. Techniques in comparative respiratory physiology—an experimental approach. Cambridge University Press, Cambridge.

Heuer, R. M., and M. Grosell. 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. American Journal of Physiology—Regulatory Integrative and Comparative Physiology 307:1061–1084.

Hickman, C. P., and B. F. Trump. 1969. The kidney. Pages 91–239 *in* W. S. Hoar, and D. J. Randall, editors. Fish physiology, volume 1. Excretion, ionic regulation, and metabolism. Academic Press, New York.

Hirano, T. M.-G., and N. Mayer-Gostan 1976. Eel esophagus as an osmoregulatory organ. Proceedings of the National Academy of Sciences USA 73:1348–1350.

Hiroi, J., T. Kaneko, and M. Tanaka. 1999. In vivo sequential changes in chloride cell morphology in the yolk-sac membrane of Mozambique Tilapia (*Oreochromis mossambicus*) embryos and larvae during seawater adaptation. Journal of Experimental Biology 202:3485–3495.

Hiroi, J., and S. D. McCormick. 2007. Variation in salinity tolerance, gill Na+/K+-ATPase, Na+/ K+/2Cl– cotransporter and mitochondrion-rich cell distribution among three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. Journal of Experimental Biology 210:1015–1024.

Hiroi, J., and S. D. McCormick. 2012. New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. Respiratory Physiology and Neurobiology 184:257–268.

Hiroi, J., S. Yasumasu, S. D. McCormick, P. P. Hwang, and T. Kaneko. 2008. Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. Journal of Experimental Biology 211:2584–2599.

Hoar, W. S. 1988. The physiology of smolting salmonids. Pages 275–343 *in* W. S. Hoar, and D. Randall, editors. Fish physiology, volume 11B. The physiology of developing fish—viviparity and posthatching juveniles. Academic Press, New York.

Holmes, W. N., and E. M. Donaldson. 1969. The body compartments and the distribution of electrolytes. Pages 1–89 *in* W. S. Hoar and D. J. Randall, editors. Fish physiology, volume 1. Excretion, ionic regulation, and metabolism. Academic Press, New York.

Houston, A. H. 1990. Blood and circulation. Pages 273–334 *in* C. Schreck and P. B. Moyle, editors. Methods for fish biology. American Fisheries Society, Bethesda, Maryland.

Hurlbert, S. H. 1984. Pseudoreplication and the design of ecological field experiments. Ecological Monographs 54:187–211.

Hwang, P. P., T. H. Lee, and L. Y. Lin. 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. American Journal of Physiology—Regulatory Integrative and Comparative Physiology 301:28–47.

Jeffrey, J. D., N. J. Bernier, and W. G. Anderson. 2022. Endocrinology. Pages 499–549 *in* S. Midway, C. Hasler, and P. Chakrabarty, editors. Methods for fish biology, 2nd edition. American Fisheries Society, Bethesda, Maryland.

Jiang, W., X. Tian, Z. Fang, L. Li, S. Dong, H. Li, and K. Zhao. 2019. Metabolic responses in the gills of Tongue Sole (*Cynoglossus semilaevis*) exposed to salinity stress using NMR-based metabolomics. Science of the Total Environment 653:465–474.

Kalujnaia, S., J. McVee, T. Kasciukovic, A. J. Stewart, and G. Cramb. 2010. A role for inositol monophosphatase 1 (IMPA1) in salinity adaptation in the euryhaline eel (*Anguilla anguilla*). Federation of American Societies for Experimental Biology Journal 24:3981–3991.

Kijewska, A., H. Kalamarz-Kubiak, B. Arciszewski, T. Guellard, C. Petereit, and R. Wenne. 2016. Adaptation to salinity in Atlantic Cod from different regions of the Baltic Sea. Journal of Experimental Marine Biology and Ecology 478:62–67.

Kolbadinezhad, S. M., J. Coimbrala, and J. M. Wilson. 2018. Osmoregulation in the Plotosidae catfish: role of the salt secreting dendritic organ. Frontiers in Physiology 9:761.

Kölsch, G., A. Krause, N. Goetz, and S. Plagmann. 2010. The salinity preference of members of the genus *Macroplea* (Coleoptera, Chrysomelidae, Donaciinae), fully aquatic leaf beetles that occur in brackish water. Journal of Experimental Marine Biology and Ecology 390:203–209.

Kozak, G. M., R. S. Brennan, E. L. Berdan, R. C. Fuller, and A. Whitehead. 2014. Functional and population genomic divergence within and between two species of killifish adapted to different osmotic niches. Evolution 68:63–80.

Kurien, B. T., and R. H. Scofield. 2006. Western blotting. Methods 38:283–293.

Kusakabe, M., A. Ishikawa, M. Ravinet, K. Yoshida, T. Makino, A. Toyoda, A. Fujiyama, and J. Kitano. 2017. Genetic basis for variation in salinity tolerance between stickleback ecotypes. Molecular Ecology 26:304–319.

Lankford, T. E., Jr., and T. E. Targett. 1994. Suitability of estuarine nursery zones for juvenile Weakfish (*Cynoscion regalis*): effects of temperature and salinity on feeding, growth and survival. Marine Biology (Berlin) 119:611–620.

Larsen, B. K., and F. B. Jensen. 1997. Influence of ionic composition on acid-base regulation in Rainbow Trout (*Oncorhynchus mykiss*) exposed to environmental hypercapnia. Fish Physiology and Biochemistry 16:157–170.

Lee, E. T. 1980. Statistical methods for survival data analysis. Lifetime Learning Publications, Belmont, California.

Lee, J. A., D. A. Collings, and C. N. Glover. 2016. A model system using confocal fluorescence microscopy for examining real-time intracellular sodium ion regulation. Analytical Biochemistry 507:40–46.

Lerner, D. T., B. T. Bjornsson, and S. D. McCormick. 2007. Effects of aqueous exposure to polychlorinated biphenyls (Aroclor 1254) on physiology and behavior of smolt development of Atlantic Salmon. Aquatic Toxicology (Amsterdam) 81:329–336.

Lowe, M. R., W. Wu, M. S. Peterson, N. J. Brown-Peterson, W. T. Slack, and P. J. Schofield. 2012. Survival, growth and reproduction of non-native Nile Tilapia II: fundamental niche projections and invasion potential in the northern Gulf of Mexico. PLoS ONE 7:e41580.

Marino, G., P. Di Marco, A. Mandich, M. G. Finoia, and S. Cataudella. 2001. Changes in serum cortisol, metabolites, osmotic pressure and electrolytes in response to different blood sampling procedures in cultured sea bass (*Dicentrarchus labrax* L.). Journal of Applied Ichthyology 17:115–120.

Marshall, W. S., and M. Grosell. 2006. Ion transport, osmoregulation, and acid-base balance. Pages 177–230 *in* D. H. Evans, and J. B. Claiborne, editors. Fish physiology, 3rd edition. CRC Press, Boca Raton, Florida.

Matey, V., J. G. Richards, Y. Wang, C. M. Wood, J. Rogers, R. Davies, B. W. Murray, X. Q. Chen, J. Du, and C. J. Brauner. 2008. The effect of hypoxia on gill morphology and ionoregulatory status in the Lake Qinghai Scaleless Carp, *Gymnocypris przewalskii*. Journal of Experimental Biology 211:1063–1074.

McCormick, S. D. 1990. Fluorescent labelling of Na^+,K^+-ATPase in intact cells by use of a fluorescent derivative of ouabain: salinity and teleost chloride cells. Cell and Tissue Research 260:529–533.

McCormick, S. D. 1993a. Methods for non-lethal gill biopsy and measurement of Na^+,K^+-ATPase activity. Canadian Journal of Fisheries and Aquatic Sciences 50:656–658.

McCormick, S. D. 1993b. Opercular membranes and skin. Pages 231–238 *in* P. W. Hochachka and T. P. Mommsen, editors. Biochemistry and molecular biology of fishes, volume 3. Analytical techniques. Elsevier, Amsterdam.

McCormick, S. D. 1995. Hormonal control of gill Na^+,K^+-ATPase and chloride cell function. Pages 285–315 *in* C. M. Wood, and T. J. Shuttleworth, editors. Fish physiology, volume 14. Ionoregulation: cellular and molecular approaches. Academic Press, New York.

McCormick, S. D. 2013. Smolt physiology and endocrinology. Pages 199–251 *in* S. D. McCormick, A. P. Farrell, and C. J. Brauner, editors. Euryhaline fishes, volume 32. Academic Press, Amsterdam.

McCormick, S. D. and H. A. Bern. 1989. *In vitro* stimulation of Na^+,K^+-ATPase activity and ouabain binding by cortisol in Coho Salmon gill. American Journal of Physiology—Regulatory Integrative and Comparative Physiology 256:707–715.

McCormick, S. D., and D. Bradshaw. 2006. Hormonal control of salt and water balance in vertebrates. General and Comparative Endocrinology 147:3–8.

McCormick, S. D., K. M. Kelley, G. Young, R. S. Nishioka, and H. A. Bern. 1992. Stimulation of Coho Salmon growth by insulin-like growth factor-I. General and Comparative Endocrinology 86:398–406.

McCormick, S. D., D. T. Lerner, M. Y. Monette, K. Nieves-Puigdoller, and B. Th. Björnsson. 2009. Taking it with you when you go: how perturbations to the freshwater environment, including temperature, dams and contaminants, affects seawater performance of anadromous fish. Pages 195–214 *in* A. J. Haro, K. L. Smith, R. A. Rulifson, C. M. Moffitt, R. J. Klauda, M. J. Dadswell, R. A. Cunjak, J. E. Cooper, K. L. Beal, and T. S. Avery, editors. Challenges for fiadromous fishes in a dynamic global environment. American Fisheries Society, Symposium 69, Bethesda, Maryland.

McCormick, S. D., A. M. Regish, A. K. Christensen, and B. T. Björnsson. 2013. Differential regulation of sodium-potassium pump isoforms during smolt development and seawater exposure of Atlantic Salmon. Journal of Experimental Biology 216:1142–1151.

McCormick, S. D. and J. M. Romero. 2017. Conservation endocrinology. Bioscience 67:429–442.

McDonnell, L. H., J. E. Reemeyer, and L. J. Chapman. 2019. Independent and interactive effects of long-term exposure to hypoxia and elevated water temperature on behavior and thermal tolerance of an equatorial cichlid. Physiological and Biochemical Zoology: 92:253–265.

McDonough, A. A., L. C. Veiras, J. N. Minas, and D. L. Ralph. 2015. Considerations when quantitating protein abundance by immunoblot. American Journal of Physiology—Cell Physiology 308:C426–433.

McGeer, J., R. Playle, C. Wood, and F. Galvez, 2000. A physiologically based biotic ligand model for predicting the acute toxicity of waterborne silver to Rainbow Trout in freshwaters. Environmental Science and Technology34:4199–4207.

McManus, L. C., S. Yurek, P. B. Teare, T. E. Dolan, and J. E. Serafy. 2014. Killifish habitat suitability as a measure of coastal restoration performance: integrating field data, behavioral trials and simulation. Ecological Indicators 44:173–181.

Milligan, C. L., and C. M. Wood. 1982. Disturbances in haematology, fluid volume distribution and circulatory function associated with low environmental pH in the Rainbow Trout *Salmo gairdneri.* Journal of Experimental Biology 99:397–415.

Moon, T. W. 1994. Measurement of intracellular calcium in fish liver cells. Pages 407–416 *in* P. W. Hochachka and T. P. Mommsen, editors. Biochemistry and molecular biology of fishes, volume 3. Analytical Techniques. Elsevier, Amsterdam.

Nishioka, R. S. 1980. Hypophysectomy of tilapia (*Sarotherodon mossambicus*) through the orbit. General and Comparative Endocrinology 40:377–378.

Nordlie, F. G. 2009. Environmental influences on regulation of blood plasma/serum components in teleost fishes: a review. Reviews in Fish Biology and Fisheries 19:481–564.

Osborn, M., and S. Isenberg. 1998. Immunocytochemistry of frozen and of paraffin tissue sections. Pages 486–492 *in* J. E. Celis, editor. Cell biology: a laboratory handbook, volume 2. Academic Press, New York.

Ou, M., T. J. Hamilton, J. Eom, E. M. Lyall, J. Gallup, A. Jiang, J. Lee, D. A. Close, S.-S. Yun, and C. J. Brauner. 2015. Responses of Pink Salmon to CO_2-induced aquatic acidification. Nature Climate Change 5:950–955.

Paquin, P., A. Redman, A. Ryan, and R. Santore. 2012. Modeling the physiology and toxicology of metals. Pages 429–484 *in* C. M. Wood, A. P. Farrell, and C. J. Brauner, editors. Fish physiology, volume 31B. Homeostasis and toxicology of non-essential metals. Academic Press, New York.

Penny, F. M., and J. D. Kieffer. 2014. Oxygen consumption and haematology of juvenile Shortnose Sturgeon *Acipenser brevirostrum* during an acute 24h saltwater challenge. Journal of Fish Biology 84:1117–1135.

Perry, S. F., and L. Rivero-Lopez. 2012. Does the presence of a seawater gill morphology induced by dietary salt loading affect Cl^- uptake and acid-base regulation in freshwater Rainbow Trout *Oncorhynchus mykiss*. Journal of Fish Biology 80:301–311.

Petzel, D. 2005. Drinking in Antarctic fishes. Polar Biology 28:763–768.

Pörtner, H., R. Boutilier, and D. Toews. 1990. Determination of intracellular pH and PCO_2 after metabolic inhibition by fluoride and nitrilotriacetic acid. Respiration Physiology 81:255–274.

Rankin, J. C., C. S. Cobb, S. C. Frankling, and J. A. Brown. 2001. Circulating angiotensins in the river lamprey, *Lampetra fluviatilis*, acclimated to freshwater and seawater: possible involvement in the regulation of drinking. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology 129B:311–318.

Reis-Santos, P., S. D. McCormick, and J. M. Wilson. 2008. Ionoregulatory changes during metamorphosis and salinity exposure of juvenile Sea Lamprey (*Petromyzon marinus* L.). Journal of Experimental Biology 211:978–988.

Richards, J. G., J. W. Semple, J. S. Bystriansky, and P. M. Schulte. 2003. Na+/K+-ATPase a-isoform switching in gills of Rainbow Trout (*Oncorhynchus mykiss*) during salinity transfer. Journal of Experimental Biology 206:4475–4486.

Rummer, J. L., and C. J. Brauner. 2011. Plasma-accessible carbonic anhydrase at the tissue of a teleost fish may greatly enhance oxygen delivery: in vitro evidence in Rainbow Trout, *Oncorhynchus mykiss*. Journal of Experimental Biology 214:2319–2328.

Sackville, M., J. M. Wilson, A. P. Farrell, and C. J. Brauner. 2012. Water balance trumps ion balance for early marine survival of juvenile Pink Salmon (*Oncorhynchus gorbuscha*). Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 182:781–792.

Samaras, A., N. Papandroulakis, M. Costari, and M. Pavlidis. 2016. Stress and metabolic indicators in a relatively high (European Sea Bass, *Dicentrarchus labrax*) and a low (Meagre, *Argyrosomus regius*) cortisol responsive species, in different water temperatures. Aquaculture Research 47:3501–3515.

Sampaio, L. A., and A. Bianchini. 2002. Salinity effects on osmoregulation and growth of the euryhaline flounder *Paralichthys orbignyanus*. Journal of Experimental Marine Biology and Ecology 269:187–196.

Sardella, B. A., D. W. Baker, and C. J. Brauner. 2009. The effects of variable water salinity and ionic composition on the plasma status of the Pacific Hagfish (*Eptatretus stoutii*). Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 179:721–728.

Sardella, B. A., and C. J. Brauner. 2007. The Osmo-respiratory compromise in fish; the effects of physiological state and the environment. Pages 147–165 *in* M. N. Fernandes, F. T. Rantin, G. M. L., and B. G. Kapoor, editors. Fish respiration and environment. Science Publisher, Enfield, New Hampshire.

Schnell, S., L. C. Stott, C. Hogstrand, C. M. Wood, S. P. Kelly, P. Pärt, S. F. Owen, and N. R. Bury. 2016. Procedures for the reconstruction, primary culture and experimental use of Rainbow Trout gill epithelia. Nature Protocols 11:490–498.

Schultz, E. T., and S. D. McCormick. 2013. Euryhalinity in an evolutionary context. Pages 477–533 *in* S. D. McCormick, A. P. Farrell, and C. J. Brauner, editors. Fish physiology, volume 32. Euryhaline fishes. Academic Press, New York.

Schwarz, D. E., and P. J. Allen. 2014. Effects of salinity on growth and ion regulation of juvenile Alligator Gar *Atractosteus spatula*. Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology 169:44–50.

Serrano, X., M. Grosell, and J. E. Serafy. 2010. Salinity selection and preference of the Grey Snapper *Lutjanus griseus*: field and laboratory observations. Journal of Fish Biology 76:1592–1608.

Shahriari Moghadam, M., B. Abtahi, S. Mosafer Khorjestan, and M. A. Bitaab. 2013. Salinity tolerance and gill histopathological alterations in *Liza aurata* Risso, 1810 (Actinopterygii: Mugilidae) fry. Italian Journal of Zoology 80:503–509.

Shelford, V. E., and E. B. Powers. 1915. An experimental study of the movements of herring and other marine fishes. Biological Bulletin 28:315–334.

Shiraishi, K., J. Hiroi, T. Kaneko, M. Matsuda, T. Hirano, and T. Mori. 2001. In vitro effects of environmental salinity and cortisol on chloride cell differentiation in embryos of Mozambique Tilapia, *Oreochromis mossambicus*, measured using a newly developed "yolk-ball" incubation system. Journal of Experimental Biology 204:1883–1888.

Smith, H. W. 1961. From fish to philosopher, 3rd edition. Doubleday, New York.

Soivio, A., K. Nynolm, and K. Westman. 1975. A technique for repeated sampling of the blood of individual resting fish. Journal of Experimental Biology 63:207–217.

Sollid, J., P. De Angelis, K. Gundersen, and G. E. Nilsson. 2003. Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. Journal of Experimental Biology 206:3667–3673.

Specker, J. L., D. M. Portesi, S. C. Cornell, and P. A. Veillette. 1994. Methodology for implanting cortisol in Atlantic Salmon and effects of chronically elevated cortisol on osmoregulatory physiology. Aquaculture 121:181–193.

Staaland, H. 1969. A device for the study of salinity preference in mobile marine animals. Comparative Biochemistry and Physiology 29:853–857.

Sucré, E., F. Vidussi, B. Mostajir, G. Charmantier, and C. Lorin-Nebel. 2012. Impact of ultraviolet-B radiation on planktonic fish larvae: alteration of the osmoregulatory function. Aquatic Toxicology 109:194–201.

Summerfelt, R. C., and L. C. Smith. 1990. Anesthesia, surgery, and related techniques. Pages 213–272 *in* C. B. Schreck, and P. B. Moyle, editors. Methods for fish biology. American Fisheries Society, Bethesda, Maryland.

Takei, Y., and S. D. McCormick. 2013. Hormonal control of fish euryhalinity. Pages 69–123 *in* S. D. McCormick, C. J. Brauner, and A. P. Farrell, editors. Fish physiology, volume 32. Euryhaline fishes. Academic Press, Amsterdam.

Takei, Y., T. Tsuchida, and A. Tanakadate. 1998. Evaluation of water intake in seawater adaptation in eels using a synchronized drop counter and pulse injector system. Zoological Science 15:677–683.

Taylor, A. 1999. Biomedical applications of atomic spectroscopy. Pages 174–182 *in* J. C. Lindon, editor. Encyclopedia of spectroscopy and spectrometry, 2nd edition. Academic Press, Oxford, UK.

Varsamos, S., S. E. W. Bonga, G. Charmantier, and G. Flik. 2004. Drinking and Na^+/K^+ ATPase activity during early development of European Sea Bass, *Dicentrarchus labrax*—ontogeny and short-term regulation following acute salinity changes. Journal of Experimental Marine Biology and Ecology 311:189–200.

Velotta, J. P., E. T. Schultz, S. D. McCormick, and R. J. O'Neill. 2014. Relaxed selection causes microevolution of seawater osmoregulation and gene expression in landlocked Alewives. Oecologia 175:1081–1092.

Velotta, J. P., J. L. Wegrzyn, S. Ginzburg, L. Kang, S. Czesny, R. J. O'Neill, S. D. McCormick, P. Michalak, and E. T. Schultz. 2017. Genomic imprints of adaptation to freshwater: parallel evolution of osmoregulatory gene expression in the Alewife. Molecular Ecology 26:831–848.

Verhille, C. E., T. F. Dabruzzi, D. E. Cocherell, B. Mahardja, F. Feyrer, T. Foin, M. R. Baerwald, and N. A. Fangue. 2016. Inter-population differences in salinity tolerance and osmoregulation of juvenile wild and hatchery-born Sacramento splittail. Conservation Physiology 4:cov063.

Wells, M. M. 1915. Reactions and resistance of fishes in their natural environment to acidity, alkalinity and neutrality. Biological Bulletin 29:221–257.

Whitehead, A., J. L. Roach, S. J. Zhang, and F. Galvez. 2011. Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. Proceedings of the National Academy of Sciences USA 108:6193–6198.

Wikelski, M., and S. J. Cooke, 2006. Conservation physiology. Trends in Ecology and Evolution 21:3846.

Wilson, J. M., P. Laurent, B. L. Tufts, D. J. Benos, M. Donowitz, A. W. Vogl, and D. J. Randall. 2000. NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. Journal of Experimental Biology 203:2279–2296.

Wilson, R. W., F. J. Millero, J. R. Taylor, P. J. Walsh, V. Christensen, S. Jennings, and M. Grosell. 2009. Contribution of fish to the marine inorganic carbon cycle. Science 323:359–362.

Wood, C. M. 1992. Flux measurements as indices of H^+ and metal effects on freshwater fish. Aquatic Toxicology 22:239–263.

Wood, C. M. 2012. An introduction to metals in fish physiology and toxicology: basic principles. Pages 1–51 *in* C. M. Wood, A. P. Farrell, and C. J. Brauner, editors. Fish physiology, volume 31A. Homeostasis and toxicology of essential metals. Academic Press, New York.

Yaacob, E. N., D. Vandenbosch, T. Coenye, A. Bajek, D. Vanrompay, K. Dierckens, and P. Bossier. 2016. Drinking activity and microparticle size selection in early post-hatching axenic European Sea Bass (*Dicentrarchus labrax* L.) larvae. Aquaculture 463:37–42.

Young, P. S., and J. J. Cech, Jr. 1996. Environmental tolerances and requirements of splittail. Transactions of the American Fisheries Society 125:664–678.

Zadunaisky, J. 1984. The chloride cell: the active transport of chloride and the paracellular pathways. Pages 129–176 *in* W. S. Hoar and D. J. Randall, editors. Fish physiology, volume 10, Gills. part B: Ion and water transfer. Academic Press, New York.

Zall, D. M., D. Fisher, and M. Q. Garner. 1956. Photometric determination of chlorides in water. Analytical Chemistry 28:1665–1668.

Zaugg, W. S. 1982. A simplified preparation for adenosine triphosphatase determination in gill tissue. Canadian Journal of Fisheries and Aquatic Sciences 39:215–217.