

Pickled Fish Anyone?

The Physiological Ecology of Fish from Naturally Acidic Waters

Jay A. Nelson

Abstract Naturally acidic freshwaters are found worldwide, yet the fishes of these habitats have been infrequently studied. Systems that formed during the Holocene tend to be depauperate of fishes, yet older, tropical acidic waters are among the most diverse fish habitats. Occupants of naturally acidic waters come from a diverse array of piscine taxa, but geographic location appears to be more important than taxon in driving final fish assemblage structure. Based on studies from relatively rapid exposure, environmental acid is predicted to challenge physiological regulation of many systems across multiple life-history stages of fish. The study of how fish deal with acidity in nature began almost a century ago, and flourished in the last third of the twentieth century as concerns over anthropogenic acidification of freshwaters grew, but has recently diminished, possibly because concerns over anthropogenic ocean acidification have taken priority. Relative to comparison species and conspecific populations, animals indigenous to acidic habitats have less disruption of monovalent ion balance under acid exposure, bind Ca^{++} more tightly at their gills or have Ca^{++} -insensitive monovalent ion efflux, exhibit greater blood oxygen transport capacity, and have exercise metabolism and performance metrics that are less affected by low pH. With the exception of ion regulation, these conclusions are based on only a handful of studies; many other potentially interesting facets of fish biology have yet to be investigated. Considering the continued acidification of freshwater and marine environments by man, further study of how fishes adapted to natural acidity will certainly bear fruit.

1 Naturally Acidic Waters of the Planet and Their Fishes

Between 1 and 3 % of the earth's surface can be classified as naturally acidic (Clymo 1984). These systems can be found in recently deglaciated regions across the temperate globe, throughout the tropics, and in regions where geological deposits or volcanism releases acid equivalents to the ecosystem. Interestingly,

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the freshwater components of these systems can be depauperate of fish fauna compared to nearby circumneutral systems (Rahel and Magnuson 1983), or they can be among the most speciose freshwater habitats on the planet (Chao 2001). Naturally acidic waters are most commonly formed through a combination of a drainage basin with either weathering-resistant bedrock or little erosion potential, resulting in very ion-poor water with minimal buffering capacity into which natural organic matter (NOM) is released by biotic processes. Incomplete oxidation of both plant and animal debris generates compounds with acidic functional groups such as humic, fulvic, and tannic acids, among others. Release of these compounds into poorly buffered water will lower the pH and, because this complex mixture of compounds also absorbs light across the visible spectrum, will generally darken the waters causing them to often be referred to as “black waters.” “Dystrophic” is a limnological term also often applied to these waters. The fishes from these systems tend to be poorly studied; the few studies come primarily from systems of Holocene origin in North America, Eurasia, Southeast Asia, and New Zealand as well as the much older Rio Negro Basin of South America. Among the temperate Holocene systems, the abundance of hydrogen ion appears to be restricting species diversity in the northern hemisphere (Amarasinghea and Welcomme 2002), but less so in New Zealand (Collier et al. 1990). In Southeast Asia, the black water systems are also of Holocene origin (Wüst et al. 2007), but are characterized by only slightly lower species diversity (Beamish et al. 2003). Considering the incredible species diversity of the older Rio Negro system (over 1,000 fish species, approximately three times the diversity of all of Europe) might lead one to assume that long-term natural acidity promotes speciation, but taken in the context of the overall fish species richness of the neotropics, this assumption would be premature. Certainly, other abiotic factors such as oxygen and temperature will also contribute to the ultimate assemblage found in any of these naturally acidic systems.

There can be little generalization concerning which fish taxa will form the piscine community of naturally acidic waters. In north temperate systems, the protacanthopterygian umbrids tend to be the most acid tolerant and occupy these systems, often as the only fish species present (Rahel and Magnuson 1983; Dederen et al. 1986; Fig. 1). The next most acid-tolerant fishes in north temperate systems tend to be advanced teleosts (percids and centrarchids; Rahel and Magnuson 1983; Rask and Virtanen 1986; Gonzalez and Dunson 1989b; Fig. 1). In New Zealand, the most tolerant fishes are also protacanthopterygian, but here it is galaxids found in the black waters. Among the other acid-tolerant fishes in New Zealand are a representative of a very ancient lineage, the jawless fishes (Geotriidae), a family of quite modern teleost (Eleotridae), and then a couple of representatives of the ancestral teleost family Anguillidae (Collier et al. 1990; Fig. 1). Likewise, for tropical systems, one cannot generalize as to which fish taxa will be found in acidic black waters. The occupants of the Rio Negro and its black water tributaries range from amongst the few freshwater elasmobranchs (Potamotrygonidae) through ancient teleosts (Osteoglossidae) up to very modern teleosts such as cichlids, with representatives from many intermediate stages of teleostean evolution. As for all Neotropical waters, the major ostariophysan lineages, characiforms, siluriforms,

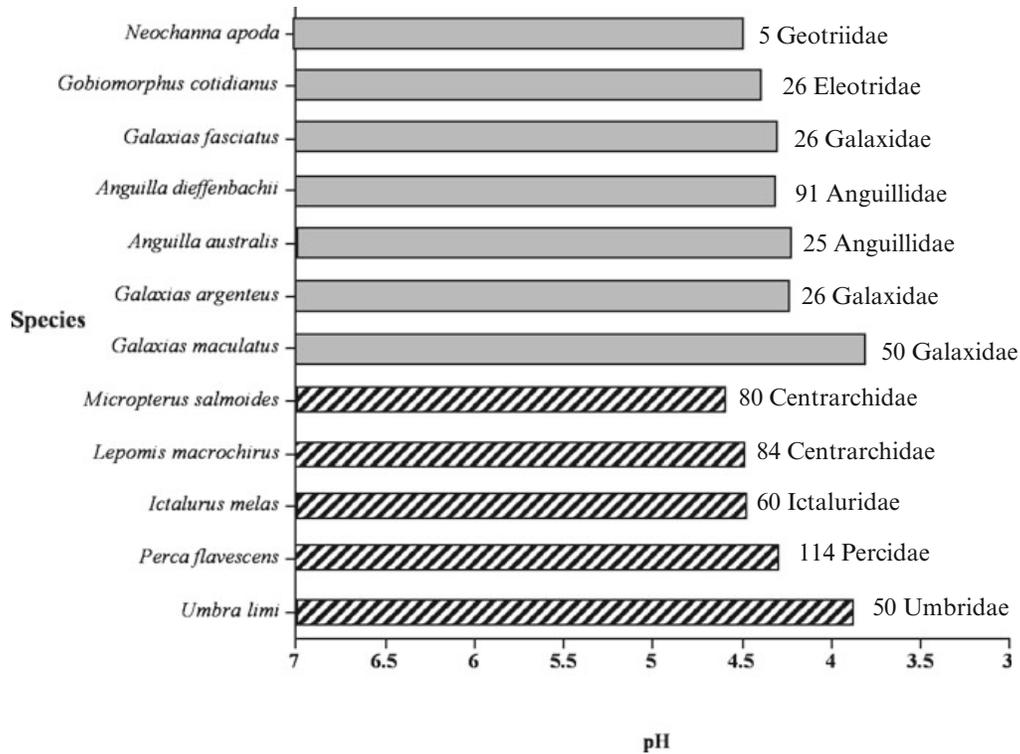


Fig. 1 Taxonomic composition of fish assemblages from naturally acidic waters in Wisconsin (USA) and New Zealand. The five most acid-tolerant fish species from Wisconsin (*hatched bars*; Rahel and Magnuson 1983) and the seven most acid-tolerant fish from New Zealand (*solid bars*; Collier et al. 1990) plotted by their pH range of occurrence. The number to the right of each bar is the number of water bodies the range was based upon followed by the family of the fish

and gymnotiforms, are well represented amongst the 1,000+ species of the Rio Negro watershed (Goulding et al. 1988). Interestingly, a group not present in the Neotropics and generally thought to be acid-intolerant from laboratory and north temperate studies, the cyprinids, make up a significant portion of the fish assemblages in naturally acidic waters on the Malay peninsula (Beamish et al. 2003) and are found in the most acid waters of Japan (Ikuta et al. 2000). Besides determining whether an animal was present as a naturally acidic system formed, taxon does not appear to be that important in determining final fish assemblage structure. Other biotic factors such as coincident species or whether a given lineage evolved air breathing could be just as important as a species' ability to tolerate acid.

2 Why Excess Hydrogen Ion in the Water Is Challenging for Fish

Of all commonly encountered ions, organisms are generally least tolerant of internal changes in $[H^+]$ (commonly expressed as the pH, or negative logarithm of $[H^+]$), so it follows that water-breathing organisms that must have thin epithelia in contact with the water for gas exchange would be sensitive to excess $[H^+]$ (acid or low pH waters) in their environment. The reason that H^+ is such a problem compared with other monovalent cations is that there are no electrons shielding the center of positive charge, in this case a naked proton, from the surrounding environment. This concentrated positive charge has a powerful destabilizing effect on polar biological molecules like proteins that depend upon weak charges to maintain the higher order structure they need to function.

The discovery of cultural acidification of freshwaters in many parts of the world in the latter half of the twentieth century, coincident with faunal changes correlating with acidification, particularly the decline of many economically important fish species, led to a voluminous literature on how environmental H^+ influences fish biology (Reviewed by Wood and McDonald 1982; Reid 1995; Kwong et al. 2014). Most of this work progressed with model system, aquaculture, or aquarium species that had not experienced low pH waters in their recent evolutionary history (i.e., acid-naïve fish). This work has taught us much about the immediate and trans-generational effects of low pH waters on fish species and populations, but little about how fish respond to acidic waters on evolutionary timescales. Water-breathing freshwater fish are hyperosmotic to their environment so that they must constantly import ions from their food and environment to compensate for diffusive ion losses, primarily from the gills that must have a large diffusive capacity to function as a respiratory organ (see Dymowska et al. 2012 for a recent review). Acid-naïve fish exposed to large environmental concentrations of H^+ generally suffer from increased mortality, net losses of essential monovalent ions such as Na^+ , Cl^- , HCO_3^- , and K^+ , are acidotic, and occasionally asphyxiate (Wood and McDonald 1982; Wood 2001; Kwong et al. 2014; Fig. 2). Asphyxiation is thought to be from excess mucus production increasing the diffusional distance for branchial oxygen uptake and is generally seen only at extremely low pH values or when (Al^{+3}) is involved (Reid 1995). Chronic effects of acid exposure can include reduced growth, metabolic alterations, gill damage, reduced skeletal ossification, behavioral abnormalities, endocrine malfunction, immune system compromise, and sensory deprivation (LeDuc et al. 2013; Kwong et al. 2014; Fig. 2). Moreover, the effects of acid exposure are sensitive to a number of environmental covariates such as $[Ca^{++}]$, $[Na^+]$, and $[Cl^-]$, total conductivity, pO_2 , pCO_2 , temperature, and the presence of various heavy metals (Wood and McDonald 1982). Of these, environmental $[Ca^{++}]$ has been singled out as the most important environmental covariate in determining fish survival in acidic fresh waters, whereas aluminum (Al^{+3}) was found to be the most significant metal ion with synergistic toxicity (Wood and McDonald 1982). The most important site of toxicity in adult fish is the fish gill,

where inhibition of ion-transport proteins in gill ionocytes and disruption of the integrity of intercellular junctions are frequently reported lesions (Reid 1995; Wood 2001; Kwong et al. 2014; Fig. 2). Investigations into acid precipitation and fish kills generally found that fish were extirpated from waters at pH levels substantially higher than were distressful to adult fish in the laboratory. There are many potential explanations for this finding. Two of the most important turned out to be: 1) that aluminum was commonly released into the waters of acidifying watersheds and had a complex synergistic toxicity that varied with pH level and dissolved organic molecules, and 2) that for most species, individuals were more vulnerable to reduced environmental pH during reproductive life-history stages (Sayer et al. 1993). Aluminum toxicity at low pH is a complex function of oxidation state, pH, environmental $[Ca^{++}]$, and dissolved organic carbon, but since it is generally a concern for fish in anthropogenically acidified waters only (Collier et al. 1990), this synergistic toxicity with low pH will not be considered further here.

A multitude of studies employing many species have reported failure of monovalent ion homeostasis in adult fish exposed to acid water (Wood and McDonald 1982; Kwong et al. 2014). Although a general finding, there remains uncertainty as to some of the mechanisms. Increased environmental $[H^+]$ is generally associated with an inhibition of both branchial Na^+ and Cl^- uptake from the water. Furthermore, acidic water increases branchial permeability to Na^+ , Cl^- , and K^+ , causing increased losses of these ions from the fish according to their electrochemical gradients. The combination of reduced monovalent ion uptake from the

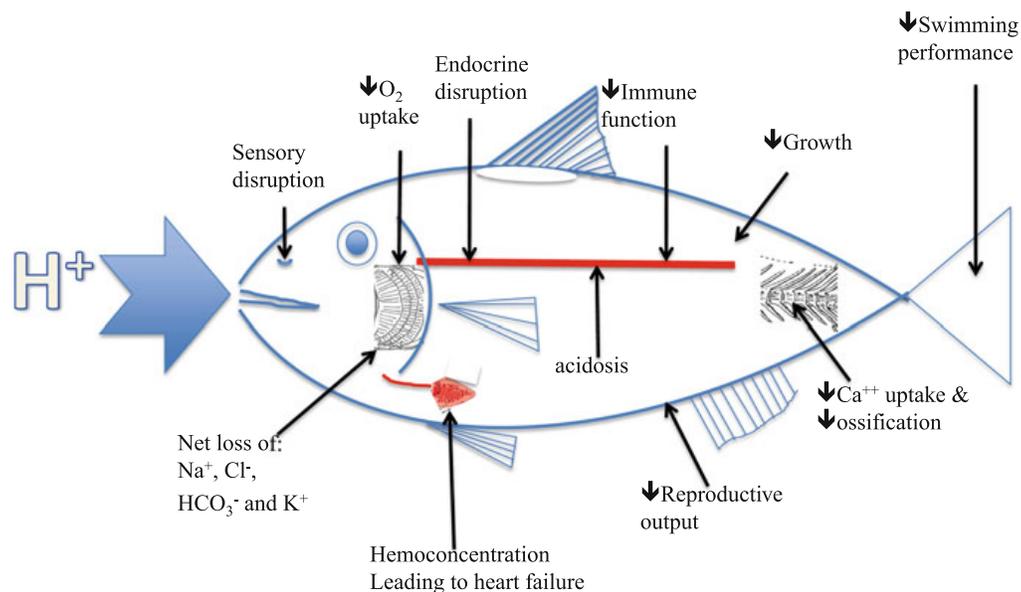


Fig. 2 Loci of documented physiological disruption by H^+ in fishes. A specific physiological lesion induced by low pH is listed with an arrow pointing to the primary place in the fish where the lesion is manifested. See text for details

environment, coupled with exacerbated losses of these ions, makes reduced plasma monovalent ion concentrations a hallmark symptom of acid exposure, but the magnitude of the reduction and the environmental pH at which it occurs vary strongly by species (McDonald et al. 1991). Environmental uptake of Ca^{++} is also limited by low pH exposure and Ca^{++} efflux accelerated, which can have significant physiological effects when the animal is not obtaining dietary Ca^{++} (Nelson 1982). The important role of environmental $[\text{Ca}^{++}]$ in determining H^+ toxicity became apparent from a number of studies showing that H^+ displaced $[\text{Ca}^{++}]$ from the gill muco-epithelial layer where Ca^{++} has the presumptive role of stabilizing tight junctions thereby limiting the diffusive loss of both Na^+ and Cl^- (Reid 1995; Kwong et al. 2014). This effect was generally dependent upon the environmental $[\text{Ca}^{++}]$, so that ionoregulatory failure was much more pronounced in softer water at a given pH (Wood and McDonald 1982). In very hard water, acidification can have negligible ionoregulatory effects, and instead acidosis becomes the primary lesion (McDonald et al. 1980), although increased renal excretion of acid equivalents can somewhat compensate for the acidosis (McDonald and Wood 1981). A number of investigators have examined gill morphology in fishes exposed to low pH or acclimating to low pH, but when the confounding effects of Al^{+++} are eliminated, there does not appear to be consistent histological changes associated with H^+ exposure unless it is severe and acute (Reid 1995). Milligan and Wood (1982) present a model for how reductions in monovalent plasma ion concentrations might lead to circulatory failure and death, but neurobiological disruptions could be just as relevant when low pH exposure is in situ (e.g., LeDuc et al. 2013) and interactions with unaffected species relevant or when eggs and larval fishes fail to mature (Chulakasem et al. 1989).

Disruptions to fish reproduction at low pH have been reported for all stages of the reproductive process from gametogenesis and spawning through hatching to egg and fry survival (reviewed in Sayer et al. 1993); for most species, one of these early life-history stages is the most vulnerable to low pH and determines the lowest pH at which the species will be found in nature. The absolute lowest pH tolerated by fish is extremely variable among species and populations; just within North American fish species, there is a three order of magnitude difference in the $[\text{H}^+]$ of their distribution (Rahel and Magnuson 1983).

The global acidification of the oceans from cultural CO_2 production has sparked a recent uptick in research on H^+ effects on marine fishes (Branch et al. 2013). Because acidification is by CO_2 , whether a given effect is from H^+ per se or hypercarbia cannot be teased out. Because of the high buffering capacity of marine waters, the pH changes due to cultural CO_2 accumulation are small; nevertheless, they have been linked to sensory, learning, and behavioral deficits (Branch et al. 2013), but nothing like the mass mortalities and fish extirpations of acidified freshwaters have been reported for marine systems. Mirroring the interest in fish from naturally acidic freshwaters that emanated from cultural acidification of freshwaters (see below), some investigators are now studying naturally hypercarbic

areas of the ocean to predict organismal and ecosystem consequences of ocean acidification (e.g., Hall-Spencer et al. 2008). Despite this interest in H⁺ effects on fishes stemming from anthropogenic acidification, surprisingly little is still known about fishes from the numerous naturally acidic systems on the planet.

3 Fish from Naturally Acidic Waters

Naturally acidic freshwaters account for up to 3 % of the world's freshwater ecosystems (Clymo 1984). Considering this abundance of acidic habitats, there is a surprising dearth of information on hydrogen ions as a selective agent in nature. The presence of fish species endemic to ancient acidic environments (Goulding et al. 1988) and the presence of species and populations in recently acidified waters that are more acid tolerant than conspecifics from more neutral waters (Rahel and Magnuson 1983; Turnpenny et al. 1987) implicate high environmental [H⁺] as an agent of acclimatization and natural selection in fish. The fauna of waters acidified in the late Holocene tend to be depauperate of fishes (Rahel and Magnuson 1983; Turnpenny et al. 1987), whereas more established naturally acidic systems (e.g., Rio Negro, Brazil) can have endemic black water species and are among the most diverse fish faunas on earth (Goulding et al. 1988). This seems to indicate that selection for life in soft, acidic waters could lead to speciation, but there is, as of yet, no direct evidence for this. To this author's knowledge, there has not been a comprehensive comparison of species diversity between Neotropical black water systems and closely matched circumneutral ones. Holocene-aged naturally acidic waters of Southeast Asia show reduced species diversity compared to nearby less acidic waters (Beamish et al. 2003).

4 History of Research on Fish from Naturally Acidic Waters

Minna Jewell initiated the scientific investigation of fish adapting to naturally acidic waters (Jewell 1922; Jewell and Brown; 1924; Brown and Jewell 1926). She first suggested that H⁺ could be important in structuring aquatic communities; this idea has been confirmed in recent years. Along with Brown (Brown and Jewell 1926), she carried out the first test of the hypothesis that fish from naturally acidic lakes might have adapted or acclimatized to their environment. They conducted a series of reciprocal transfers between fish from a naturally acidic lake and a nearby circumneutral lake. Because several species from circumneutral waters survived 40 days of enclosure in the naturally acidic lake in equal numbers to those enclosed

in the circumneutral lake, the authors concluded that direct effects of H^+ on adult fish did not limit fish use of the naturally acidic habitats of northern Michigan (USA). Their experiments were conducted mid-summer when pH levels are highest in these systems (primarily due to photosynthetic CO_2 removal from the water and lack of precipitation flushing organic acids from adjacent peat lands), and we now know that eggs and larvae are generally the most vulnerable life-history stages to the effects of low environmental pH (Chulakasem et al. 1989; Sayer et al. 1993). Had Brown and Jewell (1926) worked on larval or juvenile stages in the spring, they likely would have uncovered signs of adaptation or acclimation in these initial experiments. Interestingly, Brown and Jewell (1926) also conducted habitat choice experiments that showed animals from acidic lakes to actually prefer lower pH water. Jewell went on to show with Jobes (Jobes and Jewell 1927) that the alkali reserve (plasma pH with cells and all components of the CO_2 equilibrium removed) in brown bullhead (*Ictalurus nebulosus*) was not different between fish from naturally acidic and circumneutral lakes. Meanwhile, other groups were investigating the respiratory characteristics of animals from the Amazon basin, including those from acidic black water habitats. For example, Willmer (1934) found an inverse correlation between the magnitude of the Bohr shift (i.e., a decrease in hemoglobin's binding affinity for oxygen as a function of increasing pCO_2 or $[H^+]$) of the fishes' hemoglobin and their perceived environmental CO_2/H^+ exposure. This was an entirely logical finding, because a large CO_2 Bohr effect in high $[CO_2]$ water could potentiate O_2 loading at the gill, but this appears to be the first documented evidence for blood chemistry differences in fish based upon their history of acid exposure.

The great depression and the Second World War seem to have deflected the early tropical fish physiologists (e.g., Keys, Willmer, Carter, and others) from expanding their studies, which surely would have uncovered more interesting findings in fish from tropical black waters. Wiebe et al. (1934), studying the interaction between low pH and lethal oxygen concentration for several temperate freshwater species, made several interesting findings. Their laboratory findings of interspecific differences in how the lethal oxygen concentration for a fish changed as environmental pH changed would fairly accurately predict later published field distributions of fish as a function of pH (Rahel and Magnuson 1983). Wiebe et al. (1934) also clearly showed that the mechanism of fish death at low pH could differ based upon oxygen levels: asphyxiation at low $[O_2]$ and other causes (see above) when O_2 was plentiful.

Interest in fish from naturally acidic waters was renewed in the latter half of the twentieth century with the identification of anthropogenic acidification as the culprit in declining fisheries in many parts of the world. Dunson and coworkers pioneered the approach of using animals from naturally acidic waters to gain insight into the effects of acid water on fish. In a paper largely focusing on the decreased blood pH and body Na^+ loss of acid-naïve brook trout (*sic*) (*Salvelinus fontinalis*) exposed to low pH, Packer and Dunson (1970) also showed that brook trout from acidic streams had normal total body $[Na^+]$ when exposed to the same low pH. This lab quickly went on to show that laboratory strains of brook trout did not survive in

naturally acidic waters where other brook trout strains were resident (Dunson and Martin 1973), and to demonstrate substantial interstrain differences in acid tolerance among brook trout strains from Pennsylvania, USA (Robinson et al. 1976). Dunson's lab also branched out to study fishes from naturally acidic tropical waters at this time, showing substantial tolerance to pHs <4.0 in several characiforms native to the Neotropical black water Rio Negro. The acid resident fishes still lost substantial body Na^+ at these ultra-low pHs, but less than species native to circumneutral waters (Dunson et al. 1977).

As scientific interest in cultural acidification grew, a number of labs started attempting to produce acid-tolerant strains of fish either through long-term acclimation protocols or selective breeding for acid tolerance. These attempts produced mixed but mostly negative results. For example, Swarts et al. (1978) artificially selected brook trout for acid tolerance, but did not produce an F1 generation that was any more tolerant than the parents. Daye (1980), Lloyd and Jordan (1964), and Trojnar (1977) also recorded early attempts at producing acid-tolerant fishes that were not successful. However, Gjedrem (1980) was able to show heritable genetic variation in acid tolerance of brown trout (*Salmo trutta*), and by acclimating brown trout to pH 6.0 for 6 weeks, McWilliams (1980) was able to reduce Na^+ loss in brown trout at pH 4.0 from the usual 1 % of the total body pool per hour to 0.6 %. It is difficult to ascertain whether there was physiological compensation involved in this response, since the animals had lost 25 % of their body Na^+ during acclimation, thereby reducing the animal–environment gradient. McWilliams (1982) later went on to demonstrate that both the acid-tolerant European perch (*Perca fluviatilis*) and acid-acclimated brown trout had lower rates of sodium loss than acid-naïve salmonids. McWilliams (1983) was able to provide a partial mechanistic explanation for this result by showing that Ca^{++} (an important determinant of membrane permeability to monovalent cations) was lost from the surface of acid-acclimated brown trout gills slower in acid water than it was from acid-naïve trout gills. The acid-acclimated brown trout also had significantly reduced plasma levels of both Na^+ and Cl^- , which would again reduce the animal-environment gradient. In contrast, Swarts et al. (1978) showed no acclimation response to low pH in brook trout; animals exposed to sub-lethal low pH even tended to be less tolerant of lethal pH exposure than conspecifics held in more alkaline waters. Gjedrem and Rosseland (2012) summarize the current state of knowledge concerning genetic adaptation to anthropogenic acidification. While studies have detected considerable variation and significant heritability in the acid tolerance of salmonids, there is still no consistent finding of fish from culturally acidified rivers being more tolerant of low pH exposure (Schom 1985; Staurnes et al. 1995; Rosseland et al. 2001). Gjedrem and Rosseland (2012) conclude that the rate of cultural acidification occurred too fast in the late twentieth century for evolution by natural selection to produce more tolerant species or strains (see also chapter “Evolutionary Toxicology: Population Adaptation in Response to Anthropogenic Pollution” for a general review of population adaptation in response to anthropogenic pollution). Considering that most of these fish populations were also under considerable fishing pressure and

exposed to other environmental pollutants (e.g., aluminum), future evolution by natural selection to an anthropogenically acidified world should not be ruled out.

Concern over the impacts of anthropogenic acidification in the late 1970s fueled growth in the number of labs initiating studies into the biology of species and populations from naturally acidic systems in temperate regions, generally concentrating on electrolyte handling. Magnuson's group at Wisconsin-Madison (USA) found that yellow perch (*Perca flavescens*) from an acid lake was more tolerant of acute depressions of environmental pH and had lower body $[\text{Na}^+]$ than conspecifics from circumneutral waters (Rahel 1983). The perch from circumneutral waters survived acid challenge better after 3 weeks of acclimation to milder acidity, yet the large, significant gap in acid tolerance between these populations and those from naturally acidic waters remained (Rahel 1983). Similarly, Canfield et al. (1985) found reduced plasma osmolarity and $[\text{Na}^+]$ in largemouth bass (*Micropterus salmoides*) populations from naturally acidic lakes in Florida (USA), and McWilliams (1982) reported lower plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ in brown trout native to ~pH 5.5 water. Rask and Virtanen (1986) also reported lower plasma electrolytes in European perch from acid lakes but normal total body osmolarity. However, Vinogradov and Komov (1985) and Lyons (1982) did not find any differences in plasma $[\text{Na}^+]$ or plasma $[\text{Na}^+]$ loss rates upon acid exposure in European and yellow perch, respectively, from naturally acidic environments. Nelson and Mitchell (1992) sampled the same populations as Lyons (1982) and Rahel (1983) and using more sophisticated techniques did find significantly lower plasma $[\text{Cl}^-]$ but not $[\text{Na}^+]$ in yellow perch from a naturally acidic lake when sampled at a pH corresponding to their natural environment. The general finding of lower plasma electrolyte levels in fish from naturally acidic waters paralleled the acclimation results of McWilliams (1980), but uncertainty remained as to whether this is a physiological compensation or just a consequence of being in acid water. Several authors (e.g., Rask and Virtanen 1986; Gonzalez and Dunson 1987) reported lower electrolyte loss rates when fish from naturally acidic waters were exposed to even lower pH levels, which hints at a physiological response. Aquatic osmoregulators that move between different salinity habitats tend to osmoconform to some degree by carrying a lower plasma [ion] in freshwater than in brackish or saltwater (Nelson et al. 1996). Fish from dilute, often acidic freshwaters are known to have even lower salt levels in their blood than comparison fish from "typical" fresh waters (Mangnum et al. 1978; McWilliams 1982; Gonzalez and Dunson 1987, 1989a; Høbe 1987). The ability to tolerate low plasma ion levels in dilute, acidic water is saving energy that would otherwise be used for osmoregulation and would thus appear to be a regulatory response.

The emphasis on curbing air pollution as a way to combat anthropogenic acidification seemed to diminish interest and/or research funding for fish from naturally acidic waters in the late 1980s. Thus, except for several ambitious expeditions to the black waters of the Rio Negro to study primarily fish ionoregulation and follow-up studies by several of the participants, only sporadic information about fish from naturally acidic waters has appeared since then.

5 Characteristics of Fish from Naturally Acidic Waters

Relative to comparison species and conspecific populations, animals indigenous to acidic habitats have been reported to (1) be more tolerant of low pH levels (Dunson et al. 1977; Rahel 1983; Dederen et al. 1986; Wilson et al. 1999; Gonzalez et al. 1998); (2) have less disruption of monovalent ion balance during acid exposure (McWilliams 1982; Gonzalez and Dunson 1987, 1989a; Wilson et al. 1999; Gonzalez et al. 1998, 2002); (3) exhibit greater blood oxygen transport capacity (Rask and Virtanen 1986; Nelson et al. 1988; Wood et al. 1998); (4) have exercise metabolism and performance that are less affected by low pH (Holeton and Stevens 1978; Nelson 1989, 1990); and (5) bind Ca^{++} more tightly at their gills (McWilliams 1983; Gonzalez and Dunson 1989b; Gonzalez et al. 1998) or have Ca^{++} insensitive monovalent ion efflux (Gonzalez et al. 1998). Effective acclimatization or adaptation eventually leading to ecological speciation in naturally acidic waters probably involves many more physiological characters than these few that have been found to vary between animals native to acidic habitats and comparison animals.

6 Increased H^+ Tolerance of Fish from Naturally Acidic Waters

Populations of fish from several species isolated from naturally acidic waters have been shown to survive acute low pH challenges better than conspecifics from more neutral waters: Brook charr (*Salvelinus fontinalis*; Dunson and Martin 1973), Yellow perch (*Perca flavescens*; Rahel 1983), and European perch (*Perca fluviatilis*; Rask 1984). Gonzalez and Dunson (1989a) also found a centrarchid that occupies naturally acidic waters of North America (the banded sunfish, *Enneacanthus obesus*) to be more acid tolerant and to grow better at low pH than congeners from more neutral pH range waters. Other claims of greater acid tolerance for animals from naturally acidic waters were generally made solely on the basis of low pH tolerance or by comparison with other unrelated species.

7 Ion Regulation in Fish from Naturally Acidic Waters

Most laboratories studying fish from naturally acidic waters have focused on their ionoregulatory capabilities, almost exclusively at the whole animal or gill level. This is presumably because ionoregulatory failure is the most cited mechanism of mortality in acid-naïve fish exposed to low pH and because naturally acidic waters tend to be ion poor. Far more is known about the Na^+ , and to a lesser degree Cl^- , gill epithelial transport kinetics of fish from naturally acidic waters than any other

physiological factor. This research has shown that relative maintenance of monovalent ion balance at low pH tends to be a characteristic of fish from naturally acidic waters, but that this is accomplished through multiple means, leaving many fish from naturally acidic waters with monovalent ion flux characteristics indistinguishable from those of less tolerant species. This lack of convergence in mechanisms of monovalent ion regulation suggests that it is probably not a primary locus of adaptation to naturally acidic waters. As early as the late 1980s, studies on fish from naturally acidic waters were not making a strong case for Na^+ flux kinetics to be under strong selection. Comparisons of an acid-tolerant species (yellow perch, Percidae) with two taxonomically disparate and acid-intolerant species (rainbow trout, Salmonidae, and common shiner, Cyprinidae) suggested that a high-affinity Na^+ uptake mechanism that was weakly affected by low pH might be an important requirement for acid tolerance (Freda and McDonald 1988). Yet, comparisons of an even more acid-tolerant centrarchid (*E. obesus*) with less tolerant sunfishes (Gonzalez and Dunson 1987, 1989a) showed that *E. obesus* had a low affinity Na^+ uptake mechanism that was substantially inhibited at pH levels where the species is found in nature. Sodium efflux fared better, as both acid-tolerant yellow perch and banded sunfish had relatively low rates of Na^+ efflux that were less sensitive to environmental pH and $[\text{Ca}^{++}]$ than comparison animals (Freda and McDonald 1988; Gonzalez and Dunson 1987, 1989a, b). Wilson et al. (1999) compared the physiological response of a Neotropical callichthyid that does not occur in black water with those of two Neotropical characiforms, one a transient black water resident and the other a facultative black water resident, while undergoing a gradual reduction of their environmental water pH to 3.5. They found mortality and the magnitude of ion losses to correlate with each species' tendency to occupy black water and concluded that successful inhabitation of acid waters involves being able to avoid ionic disturbance. In these experiments, the "black water resident" characiform (tambaquí, *Colossoma macropomum*) suffered a net loss of both Na^+ and Cl^- at a relatively modest pH level of 5.0 and suffered significant declines in plasma concentration of both of these ions at a pH of 3.0, slightly lower than the lowest natural pH they are likely to see in the Amazon region (3.5) (Wood et al. 1998; Wilson et al. 1999). The "black water-resident" tambaquí for these experiments was obtained from aquaculture operations but was reported to have been occasionally exposed to acidic water (Wood et al. 1998). The source of the fish may prove important as three other characiforms captured in the Rio Negro and measured in Rio Negro water had much lower rates of monovalent ion loss at low pH (Gonzalez et al. 1998), which suggests that other organic substances in the "cocktail du jour" that are acidic black waters may be influencing monovalent ion balance. Matsuo and Val (2007) also found that humic compounds ameliorated low pH-induced Na^+ and Ca^{++} losses in a black water resident characiform, and Glover et al. (2012) found a significantly greater Na^+ uptake in a galaxid population from acidic, humic water when compared with conspecifics from neutral water, when both were measured in humic water. Interestingly, Gonzalez et al. (1998) found that ion fluxes in Rio Negro fishes were also insensitive to environmental $[\text{Ca}^{++}]$ in the range of 10–100 μM . Gonzalez and colleagues were able to obtain more

sophisticated flux measurements on some of the most acid-tolerant Neotropical species from black waters by obtaining them from aquarium dealers in North America (e.g., Gonzalez and Preest 1999; Gonzalez and Wilson 2001). Some of these fish exhibited branchial permeabilities on par with acid-intolerant species, whereas others had very unremarkable Na^+ uptake kinetics. Some of these species did exhibit monovalent transport kinetics fairly independent of pH while others again demonstrated flux characteristics that were insensitive to environmental $[\text{Ca}^{++}]$. Wood et al. (2002) captured rays of the genus *Potamotrygon* from the Rio Negro and used them to make the only known ion regulation measurements on freshwater elasmobranchs. The authors not only reported substantial monovalent ion losses at pH 4.0 that were somewhat ameliorated when the fluxes were measured in natural black water but also confirmed the conclusion from teleost fishes that extreme monovalent ion regulatory characteristics are not requisite for successful exploitation of naturally acidic waters. Because most of these comparative studies of ion regulation between fishes were made across great taxonomic divides without regard for phylogeny, they offer little information about how the ionoregulatory characteristics changed as species started to adapt to, or evolve in, naturally acidic waters. The vast differences in Na^+ influx parameters reported in Table 9.4 by Gonzalez et al. (2005) for fishes captured from the acidic Rio Negro, as well as the vast differences in branchial permeabilities reported across studies of acid-tolerant fishes, would suggest that monovalent ion balance is not limiting the exploitation of acidic habitats. Matsuo and Val (2002) also support this conclusion with an experimental design more suited to inferring evolution of monovalent ion regulation being part of the adaptive suite required to occupy naturally acidic waters. They compared monovalent cation fluxes of two *Corydoras* (Callichthyidae) species, only one of which occupies acidic black water habitats in the Amazon. In this study, the native black water species suffered a greater loss of Na^+ at low pH than did the neutral water species, although the acidophilic species did demonstrate $[\text{Ca}^{++}]$ insensitive Na^+ flux and less K^+ loss at low pH than the comparison species (Matsuo and Val 2002). These interspecific comparative studies all suggest that despite monovalent ion regulation being a primary lesion when acid-naïve fish encounter low pH waters, it does not appear to be a determining factor in the successful long-term colonization of acidic waters.

In contrast, several of the intraspecific comparative studies (e.g., McWilliams 1982; Rahel 1983; Rask and Virtanen 1986) and comparisons of an acid-tolerant North American centrarchid with one or two other centrarchids (Gonzalez and Dunson 1987, 1989a) have suggested that reducing net monovalent ion loss may be an important part of acclimatizing or adapting to naturally acidic temperate freshwaters. Kaneko et al. (1999) compared the response of a Japanese cyprinid (*Tribolodon hakonensis*) from a naturally acidic lake with hatchery-raised conspecifics (controls) when both were rapidly changed from neutral to pH 3.5 water. The control fish suffered a 35 % loss of plasma $[\text{Na}^+]$ and 33 % mortality within 24 h, whereas the acid-adapted fish lost only 12 % of their plasma $[\text{Na}^+]$ and had no mortality over 48 h. Glover et al. (2012) also showed minor improvements to Na^+ uptake kinetics in a galaxid population captured from naturally acidic waters.

A few studies using intraspecific comparisons of fish from naturally acidic waters conflict with these results. Lyons (1982) and Vinogradov and Komov (1985) showed no differences in monovalent ion loss between fish from naturally acidic waters and conspecifics from neutral waters upon acid exposure. Furthermore, Nelson and Mitchell (1992) compared plasma ion levels of yellow perch populations from a naturally acidic lake with conspecifics from neutral lakes after 16 h of swimming in acid water and found no difference between them. These latter results support the conclusion from the interspecific comparative studies (reviewed in Gonzalez et al. 2005) that adjustments to monovalent ion regulatory parameters are not a key part of acclimating or adapting to naturally acidic waters.

One problem with drawing inference from these comparative ionic balance studies is that the potential contribution of dietary ions is generally not included, a factor that may be key in nature. Even well-fed laboratory fishes, living in unchallenging ionic waters, extract much of their monovalent ion content from the diet (Bucking and Wood 2006). The role of dietary monovalent ions in preserving ion balance in dilute, naturally acidic waters is unstudied, but certainly merits attention. Wood et al. (2002) inferred as much in their study of *Potamotrygon* sp. from the Rio Negro, because their measured Na^+ and Cl^- efflux rates for this species far outstripped rates of uptake even under mildly acidic conditions. Nelson (1988) abandoned environmental Ca^{++} flux studies with perch from naturally acidic lakes when he discovered that the $[\text{Ca}^{++}]$ of their gut contents exceeded that of comparison conspecifics from circumneutral lakes, and developing oocytes had the same $[\text{Ca}^{++}]$ between lake types. Whether Ca^{++} obtained from the diet or liberated from bone can substitute for environmental Ca^{++} in stabilizing gill tight junctions has not been studied, but may account for the reported calcium independence of monovalent ion permeabilities in some of the reported species and experiments.

8 Blood Oxygen Transport

Wiebe et al. (1934) showed that the lethal oxygen levels for a number of freshwater fish species increased exponentially as environmental pH was lowered. Because of the potential for low environmental pH to lower the pH in the erythrocytes of the lamellar capillaries and shift the hemoglobin–oxygen affinity curve toward deoxyhemoglobin (Bohr and Root shifts) reducing blood oxygen uptake at the gills, many investigators have looked for this effect or compensatory responses in blood oxygen transport variables in acidified water (reviewed in Wood and McDonald 1982). Unfortunately, this large body of work has been inconclusive; while rapid exposure to extremely low pH has reproducibly been shown to asphyxiate fishes, possibly through the induction of excess mucus production, the evidence for disruptions of oxygen transport at milder, but still challenging, pH levels is equivocal (Wood and McDonald 1982). Similarly, changes to blood oxygen transport variables such as blood [hemoglobin], hematocrit, and erythrocyte number in

fish exposed to acid water are quite variable across studies (reviewed in Wood and McDonald 1982). Many of the changes reported could not be separated from cell swelling expected from plasma ion losses or adrenergic activation of erythrocytes (Milligan and Wood 1982). If oxygen loading is being compromised in the gills of fish from low pH waters, we would expect to see some compensation for this in fish spending thousands to millions of generations in naturally acidic systems. Indeed, Rask and Virtanen (1986) found that European perch from a naturally acidic lake had a higher hematocrit than conspecifics from a circumneutral lake; even more interesting was that the acidic lake perch increased their hematocrit in response to reductions in pH, without changes in osmolality, whereas the neutral lake perch did not (Rask and Virtanen 1986). Nelson et al. (1988) found blood sampled from yellow perch immediately after capture from a naturally acidic lake to have significantly higher [hemoglobin], hematocrit, and mean cell [hemoglobin] than blood from conspecifics in neutral lakes. Since hemoglobin is also the principal blood buffer in fishes, these results do not necessarily accrue from difficulties with oxygen transport. In contrast, McWilliams (1982) found no increase in [hemoglobin] but greater hematocrit and red blood cell number in brown trout from a hatchery with acidic water when compared with conspecifics from a neutral water hatchery, but these were concomitant with significantly lower plasma [ion] in the acid hatchery fish. Reports on blood chemistry of acid-tolerant fishes without comparison conspecific animals range from extremely high hematocrits in an air breathing mudminnow (*Umbra pygmaea*; Dederen et al. 1986) to extremely low hematocrits and [hemoglobin] in the tambaquí (Wood et al. 1998). In both studies, blood oxygen variables were relatively unaffected by reductions in pH, although the tambaquí registered increases in erythrocyte [adenylate] which would facilitate hemoglobin unloading of oxygen in the tissues and likely signals the lack of any impairment of branchial oxygen loading at low pH.

9 Blood Acid–Base Status

There is virtually no information on blood acid–base regulation of fish from naturally acidic waters. Nelson and Mitchell (1992) found that yellow perch from a naturally acidic lake carried a higher plasma pH, $[\text{HCO}_3^-]$, and base excess than comparison conspecifics from two circumneutral lakes in neutral water. Base excess is an indicator of the metabolic acid or base added to the blood and is an approximation of the “ Δ strong ion difference” (Stewart 1981; Nelson and Mitchell 1992). The “strong ion difference” theory posits that pH and $[\text{HCO}_3^-]$ are secondary variables in a solution that respond to changes in the concentrations of primary mineral ion variables (Stewart 1981). Upon exposure to acid water while swimming at 3 cm s^{-1} , the acid-lake perch showed increases in both $[\text{HCO}_3^-]$ and base excess. In contrast, the two acid-naïve populations registered decreases in plasma $[\text{HCO}_3^-]$ and base excess while plasma pH was constant across all animals and treatments. The higher average hemoglobin concentration in yellow perch from a naturally

acidic lake (Nelson et al. 1988) could account for some of the increase in plasma $[\text{HCO}_3^-]$ found by Nelson and Mitchell (1992), but higher bicarbonate and base excess after 16 h of mild exercise and acid exposure more likely accrued from differences in acid equivalent production, storage, excretion, or gill permeability. Muscle buffering capacity is the same across these yellow perch populations (Nelson and Magnuson 1987), so differential storage in the large white muscle mass is not involved; however, differential storage of H^+ equivalents in bone is possible. The higher $[\text{HCO}_3^-]$ and base excess in perch from naturally acidic lakes could also be a consequence of lower plasma chloride levels. The constraint of electrical neutrality dictates that changes of any plasma anion will result in equimolar changes of other anionic species in the opposite direction, or cationic species in the same direction. Thus, by one view, the lower Cl^- levels “generated” the additional HCO_3^- by increasing the “strong ion difference” (Stewart 1981). Ikuta et al. (2000) report on populations of a cyprinid (*Tribolodon hakonensis*) that occupy acidic lakes in Japan and have enlarged gill ionocytes with a high V-ATPase activity, suggestive of enhanced H^+ excretory capacity. A population of this same species had a much less severe plasma acidosis than conspecifics from neutral waters when both were exposed to pH 3.5 (Kaneko et al. 1999). In addition, Wood et al. (1998) showed minimal changes in blood pH and $[\text{HCO}_3^-]$ in the facultative black water resident tambaquí upon exposure to gradual pH reductions. Certainly, these few studies are indicative of successful acid/base regulation in fish from naturally acidic waters exposed to acid water but are insufficient to form any conclusions as to what, if any, modifications of this regulatory system were necessary to occupy these habitats. Understanding whether changes in the acid/base regulatory system are integral to acclimatization, adaptation, or even speciation in acidic habitats will require more comprehensive evolutionary physiological studies of acid/base regulation under environmentally realistic conditions such as swimming in acid waters.

10 Swimming Performance

Although naturally acidic freshwaters present several regulatory challenges to aquatic water-breathing vertebrates, Darwinian fitness in these environments will be determined by more than the ability to regulate ions or maintain acid/base balance. Little is known about how fish populations or species indigenous to acidic water compensate for the additional challenges inherent from essential life activities such as swimming or reproduction.

Adopting the terminology of Fry (1971), swimming activity in fish is controlled by factors such as temperature (Beamish 1978) and morphology (Webb 1975), but is limited by factors such as oxygen, carbon dioxide, pollutants, and disease (Fry 1971; Beamish 1978). Presumably, hydrogen ions are also a limiting factor for most fish, but this has not been adequately tested. The results from acid-naïve fishes generally show reduced endurance swimming ability, but not until the pH levels are

fairly extreme. For example, Waiwood and Beamish (1978) found no effect on critical swimming speed (U_{crit}) when the pH was reduced to 6.0 in rainbow trout, but West and Garside (1986) found a significant depression of U_{crit} at pH 5.0–6.0. Graham and Wood (1981) found a linear decrease in U_{crit} as pH was progressively depressed below pH 6.0 in the same species. Fish from naturally acidic waters have been shown to have their swimming performance less diminished by acid water, but this result is context dependent: Nelson (1989) reanalyzed the data reported by Holeton and Stevens (1978) to show that *Tripurtheus angulatus* (Characiformes) acclimated to white water and swimming in black water had reduced critical swimming speeds, whereas fish acclimated to black water and swimming in black water had U_{crit} values similar to controls. This was similar to the result Nelson (1989) obtained for a perch population isolated for 2,000–3,000 generations in a naturally acidic lake in northern Wisconsin (USA). These animals did not have higher absolute swimming speeds in acid water, but their swimming performance was less affected by soft, acid water than it was in conspecifics from circumneutral waters. Swimming in dilute, acid water greatly increased the range and coefficient of variation of critical swimming speeds, meaning that isolated individuals were capable of performing at the same level in acid water, yet many other individuals had their swimming ability impaired by acid water (Nelson 1989).

11 Metabolism

There is very little information about metabolic divergence along environmental hydrogen ion gradients at the population or species levels in fish. Nelson and Magnuson (1992) compared the metabolic storage patterns of yellow perch from one naturally acidic lake with two conspecific comparison populations across a single calendar year. They found no metabolic evidence of environmental acid influence. Acid-stressed fish were predicted to have higher liver [glycogen] and lower muscle [glycogen] (Dheer et al. 1987), yet the perch from the acid lake had significantly higher muscle [glycogen] and significantly lower liver [glycogen] than the perch populations from circumneutral waters (Nelson and Magnuson 1992). Furthermore, Nelson and Magnuson (1992) found the acid-lake perch to have normal condition factors and significantly higher visceral and hepatic lipid levels as well. Overall, these results were consistent with preferential use of metabolic pathways that would produce less endogenous H^+ , but were also consistent with a shifting life-history strategy not concordant with density-driven changes alone (see below).

Nelson (1990) also examined metabolic profiles of yellow perch populations, one from a naturally acidic lake, after exercise in acidic or neutral water. Acid-lake perch generally had significantly less muscle metabolic disturbance after exercise to behavioral exhaustion when compared with conspecifics from neutral lakes swimming in neutral water. This phenomenon was demonstrable via muscle concentrations of lactate, adenosine tri-phosphate, creatine phosphate, adenosine mono-

phosphate, and inosine mono-phosphate and suggested that fish that pushed adenylate depletion and glycolysis to extremes had been selected against in this population. The pH of the water had no effect on muscle metabolite accumulation during exercise or recovery in acid-lake perch. In contrast, acid-naïve perch had dramatic differences in muscle metabolite concentrations when swimming to behavioral exhaustion in different pH waters. This disruption of normal muscle metabolism was only evident at behavioral exhaustion; metabolite levels were similar during recovery from exercise, regardless of environmental pH. The acid-naïve fish were given 16 h to acclimate to the low pH water before the tests, so whatever physiological adjustments allowed the acid-lake perch to not suffer the same disruption in muscle metabolism as the acid-naïve perch were not rapidly inducible (Nelson 1990). The author also inferred from metabolite concentrations the apparent relative activities of metabolic pathways that are either proton producing or consuming. When there were noticeable differences between the acid-lake and circumneutral lake perch populations, the acid-lake perch demonstrated greater proton economy for four out of five metabolic pathways. While these results suggest that high concentrations of environmental H^+ may drive changes in fish metabolism, there is clearly room for more research in this area.

12 General Biology

Considering the prevalence of naturally acidic habitats on earth (Clymo 1984), other than their ionoregulatory characteristics, we know surprisingly little about the fishes that occupy them. Since early life-history stages are generally the most vulnerable to acidic water (Sayer et al. 1993), much of the acclimatization to low pH is likely occurring during development, and adaptive changes are likely to affect early developmental stages as well. Nelson (1988) observed massive egg mortality in yellow perch from a naturally acidic lake in a year when nearby populations from circumneutral lakes were hatching successfully. This would imply that mortality selection for acid tolerance is ongoing in yellow perch in this Holocene acidic ecosystem. How successful reproduction is in the many tropical fishes endemic to acidic waters is unknown to this author. While studies of fish reproduction in low-ionic-strength low-pH waters (e.g., Chulakasem et al. 1989) point to severe reproductive challenges for tropical black water fishes, these fishes seem to thrive, and water chemistry-induced selection on reproductive characters may be driving life-history changes. Nelson and Magnuson (1992) found greater reproductive investment in female perch from naturally acidic lakes; this result coupled with lower growth rates, but normal condition factors and greater visceral lipid stores suggested a shift toward earlier and greater reproductive investment in fish from acid waters. Some fish may even be using their level of acid tolerance as a way to take refuge from less acid-tolerant predaceous fishes (Olsson et al. 2006).

13 Conclusions and Outlook

The overwhelming conclusion from this review is that fish from naturally acidic systems merit far more study. Today, we cannot even generalize as to whether acidic habitats promote or restrict piscine diversity nor do we understand what the biological characteristics are that allow some species to thrive in acidic habitats. Considering the fact that we find jawless lampreys and ancestral teleosts among the fauna of naturally acidic systems and that the earth has experienced prior massive acidification events (e.g., Permian-Triassic boundary, 250 MYA; Ogden and Sleep 2012) within the evolutionary history of today's fishes, we would expect the ability to occupy acidic habitats to be plesiomorphic. This may explain why we find so many diverse fish taxa in extant acidic ecosystems, but the question then becomes, why have so many fishes lost the ability to thrive in acid waters? Minna Jewell (1922) postulated almost a century ago that because of the physiological challenges posed by high environmental $[H^+]$, species and populations occupying naturally acidic waters were likely to be special in some way. Today, interest in animals from acidic ocean seeps is surging as scientists seek to predict organismal and ecosystem consequences of human-generated ocean acidification, yet we still know little about how fishes deal with the more extreme acidity they encounter in naturally acidic freshwaters. Understanding how fish acclimatize to, adapt, or even speciate in acidic habitats will not only enrich our knowledge of freshwater fish evolution but may also give us a window into understanding how fish will respond to further anthropogenic acidification of freshwaters and oceans.

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