Acid-base Balance

INTRODUCTION

Regulation of intra- and extra-cellular pH (pHi and pHe, respectively) is crucial to the normal functioning of all vertebrates, including fish. Deviations in pH alter the local charge on proteins affecting enzyme function and membrane channel properties, which ultimately impact upon cell-cell signalling, cell-volume regulation, gene expression, contractility of the heart and skeletal muscles, and whole animal metabolism (reviewed by Putnam & Roos, 1997). Consequently pH regulation is one of the most tightly controlled processes and not surprisingly a great deal is known about the mechanisms involved in acid-base regulation in fish (Heisler, 1984, 1993; Clatborne et al., 2002; Evans et al., 2005). Most of this knowledge, however, comes from juvenile and adult fish and our understanding of the mechanisms and patterns of acid-base regulation during development in fish is very limited. This chapter will briefly review acid-base regulation in juvenile and adult fish, discuss the limited literature on acid-base regulation in developing fish, and provide a framework for future studies required to advance our understanding of acid-base regulation in developing fish; a field ripe for investigation.

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MECHANISMS OF ACID-BASE REGULATION IN JUVENILE AND ADULT FISH

Under control steady-state conditions, most fish exhibit a small whole body net acid excretion, as is observed in mammals, which is required for pH homeostasis. Acid-base status in fish can be influenced by a wide variety of conditions, including changes in the external environment, such as gas tensions (CO₂, NH₃, and/or O₂ levels), temperature, salinity and water pH, and internally induced challenges associated with processes such as feeding and exercise (predator avoidance, prey capture, migration). Acid-base disturbances can loosely be grouped into two categories. The first is respiratory in origin, where changes in pH are associated with changes in blood CO₂ levels. The second is metabolic in origin, and is a result of non-CO₂ related changes in pH, as occurs during anaerobic metabolism. In many instances, acid-base disturbances may be both respiratory and metabolic in origin. For example, exposure to hypoxia results in hyperventilation, inducing a respiratory alkalosis that may be combined with a metabolic acidosis due to inadequate O₂ delivery to the tissues and subsequent anaerobic metabolism. The majority of acid-base disturbances experienced in fish involve an acidosis, and consequently compensation for an acidosis will represent the main emphasis in this review. Experimentally, exposure to elevated environmental CO₂ (hypercapnia) is one of the most common means to induce an acidosis through which the pattern and process of pH recovery can be followed. This is because CO₂ can easily be maintained at a pre-determined, constant environmental level over the exposure duration, and it diffuses rapidly from the water into the fish to induce an acidosis. During an acid-base disturbance, pH changes can be buffered or actively restored through a number of pathways as described below.

Whole Animal Acid-base Regulation

In vertebrates in general, acid-base disturbances can be compensated by: i) physicochemical buffering with bicarbonate and non-bicarbonate buffers, ii) changes in ventilation to alter pCO₂ and thus pH, via the CO₂-HCO₃⁻ buffer system, or iii) net transport of acid-base equivalents between the body and the environment. In contrast to air-breathers, the partial pressure of CO₂ in the blood of water breathing fish is low due to the high ventilatory requirements to secure O₂ uptake from water (Dejours, 1988), and thus compensatory strategies i) and ii) have relatively little effect on blood pH regulation. Consequently, disturbances in blood pH in fish are generally compensated for by transepithelial transfer of acid-base relevant ions between the animal and the aquatic medium. The gills, intestine and kidney have all been implicated to some degree in acid-base regulation in fish, however, it is generally accepted that the gills are the dominant surface (about 90%) across which net acid-base transfer occurs (Heisler, 1984, 1993). The gills represent a large surface area that is in intimate contact with the aquatic medium. The gill epithelium is provided with high water flow rates, and the aquatic medium can act as an unlimited source and sink for acid-base equivalents and respective counter ions, provided that the composition of the aquatic medium is appropriate (i.e. not too low in ionic composition or acidic). In general, the rate and degree of pH compensation is influenced by ionic composition of water, where fish in hard freshwater (FW) compensate more completely and rapidly than fish in soft FW (Larsen & Jensen, 1997), and those in seawater (SW) more completely and rapidly than those in FW (Toews et al., 1983).

In general, recovery of blood pH following an acid-base disturbance results from adjustments in blood HCO₃⁻ levels (Evans et al., 2005). In studies measuring net acid excretion during an acidosis, it is impossible to distinguish between net H⁺ excretion and HCO₃⁻ uptake (Heisler, 1984). In most fish investigated to date, plasma HCO₃⁻ elevation during an acidosis is associated with a 1:1 reduction in plasma Cl⁻, implicating net branchial Cl⁻/HCO₃⁻ exchange (Toews et al., 1983; Cameron & Iwama, 1989; Larsen & Jensen, 1997) as the compensatory mechanism. Some species appear to utilise net branchial Na⁺/H⁺ exchange (Hyde & Perry, 1989; Hirata et al., 2003) to compensate for acidosis, however, this pathway appears to be much less common. In a recent study on two species of marine teleost fishes, Japanese flounder (Paralichthys olivaceus) and yellow tail (Seriola quinqueraudata), during exposure to 1% CO₂, the elevation in plasma HCO₃⁻ was matched with a similar reduction in plasma Cl⁻, however at higher CO₂ tensions of 3-5% CO₂, the rise in HCO₃⁻ exceeded the fall in Cl⁻. Interestingly, under these conditions there is an increase in plasma Na⁺ concentration that makes up most of the increase in plasma HCO₃⁻ not accounted for by a reduction in Cl⁻. Thus, in some marine fish, there appears to be a biphasic response likely involving net branchial Cl⁻/HCO₃⁻ exchange at low levels of hypercapnia, coupled with net branchial Na⁺/H⁺ exchange at higher CO₂ levels (Hayashi et al., 2004).

While most fish are capable of regulating blood pH back to close to pre-disturbance levels during an acid-base disturbance within hours to days (Utltsch, 1996), provided the acidosis is not too severe, a few species possess a very blunted extracellular acid-base regulatory response (Heisler, 1982; Brauner et al., 2004; Sanchez et al., 2005). During exposure to environmental hypercapnia in the facultative air-breathing salmonid catfish (Lipotes przewalski), blood pH may be reduced by up to 1 pH unit, with minimal pH compensation over the following 96 h, despite 100% survival. In both the salmonid catfish and another facultative air-breathing fish, the marble swamp eel (Symbanchus marmoratus), the lack of compensation for a reduction in plasma pH is associated with complete preferential pHi regulation of heart, liver and muscle (Heisler, 1982; Brauner et al., 2004). In most fish species investigated to date, pH is regulated back to pre-disturbance levels; however, this usually requires significant recovery in plasma pH for this to be accomplished (Brauner et al., 2004). The mechanisms responsible for complete preferential pH regulation in the absence of pH compensation, and the ubiquity of this pattern of acid-base regulation among fish species remains to be investigated. It is not known whether larval stages of these species exhibit this same strategy in acid-base regulation.
Cellular Processes and Transporters Associated with Acid-base Regulation

Earlier studies on acid-base regulation primarily focused on whole animal responses and net acid-base status following alterations in primarily extracellular, but also intracellular pH. While it is now well established that net adjustments in plasma HCO₃⁻ during an acid-base disturbance are associated with Na⁺ and/or Cl⁻ linked acid-base transport in both FW and SW fish (Evans et al., 2005), there are differences in both the cellular and sub-cellular pathways involved in the gills, and in the types of transport proteins used in the gills of different fishes. These differences may be species specific, and may be affected by the ambient environment, such as water pH and salinity. While recently there has been considerable interest in elucidating the cellular, sub-cellular and molecular pathways through which acid-base regulation is accomplished (reviewed by Claiborne et al., 2002 and Evans et al., 2005), there remain large gaps in our understanding of the processes involved in pH regulation in juvenile and adult fish. Not surprisingly, much less is known in this regard for larval fishes.

In general, the mitochondrial rich cell (MRC, also commonly referred to as the chloride cell, see Kaneko & Hiroi, this volume) of both FW and SW fishes is thought to be the primary cell associated with acid-base regulation. However, there is also evidence for the pavement cells in FW fish playing a role. Two distinct sub-types of MRCs exist in freshwater fish, an α sub-type, and a β sub-type (Pisam et al., 1987). One is thought to be a net acid excreting cell and the other a net base secreting cell (Calves et al., 2002; Evans et al., 2005) as is observed in the mammalian kidney.

There are two general models for net Cl⁻/HCO₃⁻ exchange across the gills during an acidosis associated with hypercapnia in freshwater. The first incorporates direct uptake of HCO₃⁻ from the water in exchange for Cl⁻ (Claiborne & Heisler, 1986; Larsen & Jensen, 1997), presumably across a branchial anion exchanger. This model is supported by the observation that an elevation in water HCO₃⁻ at constant pCO₂ results in a more rapid and complete compensation in blood pH (Larsen & Jensen, 1997) than in waters with lower HCO₃⁻ levels. The second model is based upon morphological changes in the gill filament associated with hypercapnia exposure. Within several hours to days following exposure to environmental CO₂, there is a physical covering of MRCs by pavement cells, reducing the MRC fractional area that is correlated with a reduction in branchial Cl⁻ influx (Goss et al., 1992). These morphological changes are associated with an increase in net acid excretion and retention of plasma HCO₃⁻. The net acid excretion is hypothesised to occur either by removal of Cl⁻/HCO₃⁻ exchangers from the MRCs and/or insertion of proton pumps into the pavement cells (Goss et al., 1999). These two models for net HCO₃⁻ accumulation in the plasma during hypercapnia are not mutually exclusive and it may be that both operate under different environmental conditions or developmental stages within a species, or possibly even simultaneously within an individual.

Characterisation of the pathways responsible for acid-base regulation in the gills of fish at the level of protein transporters, has been accomplished through the use of immunohistochemistry, pharmacology and molecular biology which have been the focus of a few recent reviews (Claiborne et al., 2002; Evans et al., 2005). Given that acid-base relevant ion transfer is associated with Na⁺ and/or Cl⁻ exchange, acid-base regulation is integrally coupled with ionoregulation. Consequently, models of ionoregulation in FW and SW (Perry, 1997; Marshall & Bryson, 1998; Marshall, 2002; Evans et al., 2005) are crucial to understand the molecular and cellular basis for acid-base regulation. The emerging consensus is that there are two acid secreting mechanisms in fish that are linked with Na⁺ transport. The first appears to be restricted to FW fish and relies upon apical V-type ATPase that generates an electrical gradient to drive Na⁺ uptake through an apical sodium channel (based upon studies largely limited to salmonids; Perry, 1997). The second is an apical electroneutral Na⁺/H⁺ exchanger that operates in some FW (Hirata et al., 2003) and likely most SW teleost fish (Claiborne et al., 2002). Processes related to base excretion, and thus net branchial Cl⁻/HCO₃⁻ exchange are less well understood, which is interesting given that under most acid-base disturbances, this is thought to be the primary route through which acid-base disturbances are corrected. In general it is thought that Cl⁻/HCO₃⁻ exchange occurs across an apical anion exchanger (AE1) or pendrin (Piermarini et al., 2002), but clearly much remains to be investigated in juvenile and adult fish, let alone developing fish.

MECHANISMS OF ACID-BASE REGULATION IN EARLY LIFE STAGES

Mass-specific metabolic rate scales inversely with body size in vertebrates (Schmidt-Nielsen, 1975). Given the net acid production associated with metabolism, smaller animals must have high mass specific acid excretion. Furthermore, considering the dramatic developmental changes that occur, many of which are likely pH sensitive, acid-base homeostatic processes must be operational in early life stages. However, very few studies have focused upon the ontogeny of acid-base regulation in fish, and surprisingly little is known from the period following fertilisation through to larvae.

Whole Animal Acid-base Regulation Shortly Following Fertilisation

In one of the few studies investigating acid-base regulation shortly after fertilisation, Molich and Heisler (2005) observed that in zebrafish (Danio rerio) embryos, exposure to hypercapnia resulted in a rapid acidification of the embryo. Exposure to a 10 fold elevation in water pCO₂, from 0.097 to 0.99 kPa (1% CO₂), or from 0.32 to 3.2 kPa (3.5% CO₂), acidified both the interstitial and intracellular fluid, with maximal reductions in pH achieved within about 10 minutes. As surface area to volume ratios decrease with growth, the time at which maximal pH reduction occurs during exposure to hypercapnia may increase, but because of the high diffusivity of CO₂ in water and biological membranes, equilibrium between the environment and intracellular fluid would always be expected in the order of minutes rather than hours in water-breathing
vertebrates. In the 10 to 256 cell zebrafish embryo preparation used by Molich and Heisler (2005), pH disturbances in both the interstitial and intracellular compartments associated with exposure to 1% CO₂ were largely compensated for within 2 h, presumably due to active pH regulatory processes associated with net HCO₃⁻ accumulation in both compartments. HCO₃⁻ accumulation in these compartments must be associated with acid-base relevant ion transfer between the embryo and the environment as in adult fish, but appears to occur at a much higher rate. Nothing is known of the specific mechanisms involved. It should be mentioned that in this study, the bathing medium in which the embryo was held was similar in composition to the interstitial fluid which differs markedly from FW, and this may have influenced the dynamics of acid-base regulation in that experimental setup. At higher pCO₂ levels (3.5%), pH compensation also occurred, but to a greater extent intracellularly than interstitially. From this study, it is clear that embryos are capable of regulating cellular and interstitial pH quite tightly very early in development. Rearing embryos at high pCO₂ (3.3 vs 0.3 kPa) for 24 h shortly following fertilisation, had no affect on mortality or growth (Molich & Heisler, 2005). Longer exposure durations were not conducted in this study, but would be very interesting given that chronically elevated CO₂ levels of this magnitude have been shown to reduce growth and feed conversion over longer term exposure in juvenile spotted wolfish (Anarhichas minor) (Foss et al., 2003), Atlantic salmon (Salmo salar) (Fivelstad et al., 1998), and white sturgeon (Acipenser transmontanus) (Crocker & Cech, 1996).

**Whole Animal Acid-base Regulation Following Hatch**

Although very few physiological studies have investigated the ontogeny of acid-base regulation in fish, there have been a number of studies investigating the ontogeny of ionoregulation (see Kaneko & Hiroi, this volume). These studies may shed important insights into the development of acid-base regulatory processes because of the tight interaction between pH and ion regulation. Active uptake of Na⁺ from the water (unidirectional Na⁺ uptake, Jₜ Na⁺) in developing salmonid embryo's is very low up to hatch (Rudy & Potts, 1969; Eddy & Talbot, 1985; Brauner & WOOD, 2002a, b), but then increases almost exponentially following hatch which correlates with a large increase in whole body Na⁺, K⁺-ATPase activity (NKA) activity (Fig. 1; Brauner & WOOD, 2002a). During yolk-sac absorption, Jₑ Na⁺ levels far exceed that of juvenile and adult fish until swim-up, at which time Jₑ Na⁺ have been reduced to adult levels (Brauner et al., 2003). During development, there is a progressive increase in whole body Na⁺ content (a three-fold increase between hatch and swim-up), indicating that there is significant net Na⁺ uptake from the environment. Interestingly, the same relationship is not seen for whole animal Cl⁻ dynamics, where Jₑ Cl⁻ is much lower than Jₑ Na⁺ (Misiaszek, 1996) and between hatch and swim-up there is no significant change in whole body Cl⁻ content. During development, the yolk represents a considerable store for both Na⁺ and Cl⁻, but appears to be more significant for Cl⁻ as yolk concentrations are initially 4-fold higher for Cl⁻ than Na⁺. Assuming these data indicate a limited ability for Cl⁻ exchange between the developing organism and the environment, this has very interesting implications for acid-base regulation during development given that in most FW fish, acid-base compensation in the blood compartment is associated with a net Cl⁻/HCO₃⁻ exchange. While Jₑ Na⁺ at the gills is much greater than Jₑ Cl⁻ during development, it is also in excess of that required for homeostasis (Misiaszek, 1996). Taken together these data may indicate that acid-base regulation at this stage of development may be more dependent upon net Na⁺/H⁺ exchange than Cl⁻/HCO₃⁻ exchange, and then switches to the latter some time later in development. Another possibility is that rather than a net acid-base exchange between the organism and the environment to correct for an acid-base disturbance, there is net exchange between the developing fish and yolk-sac contents. Clearly more work is required to investigate these possibilities.

There are also very interesting dynamics in relation to ammonia production and accumulation in developing embryos that may be of importance as a temporal buffer system. During development, there appears to be a progressive increase in embryonic and yolk total ammonia up to hatch, following which ammonia levels quickly decrease (reviewed by Wright and Rybnik, 2001). NH₃ is produced as an end product of protein
and amino acid catabolism. Up to and following hatch, developing fish have an ammonia quotient (mol ammonia excretion/mol oxygen consumption) of between 0.10 and 0.30. The pH, for ammonia is between 9.0 and 9.5, and thus at the pH of various body compartments, at least 95% of the total ammonia will be as NH₄⁺. Consequently, as total ammonia accumulates, it will bind and store protons. Whether ammonia accumulation in the body and yolk is important for acid-base balance in embryonic and larval fish is not known, but worthy of further study.

**Cellular Processes and Transporters Associated with Acid-base Regulation**

In adult fish, the gills are clearly the predominant structure for acid-base regulation (Heisler, 1984, 1993). However, early in development, functional gills do not exist and therefore cannot be involved. From the discussion above based upon a single study, it appears that fish embryos have the ability to regulate acid-base status very effectively shortly after fertilisation. Given that acid-base regulation is tightly linked with ionoregulation, and at the cellular level, acid-base relevant ion exchange is largely associated with MRCs and NKA, studies documenting the relative distribution of MRCs and NKA over the body surface throughout development may yield important insight into the ontogeny of acid-base regulation.

It is well documented that MRCs exist on both the skin and yolk-sac epithelium of developing fishes (Alder, 1988; Hwang, 1989; Hwang et al., 1994, 1999; Chang et al., 2003; Varsamos et al., 2005; Kaneko & Hiroi, this volume), and MRC density on these structures increase with development prior to gill formation (Rombough, 1999). It is hypothesised that skin and yolk-sac epithelium MRCs play an important role in osmoregulation until gill MRCs become functional (Kaneko et al., 2002), but this is largely based upon morphology, where there appears to be considerable homology between skin and yolk-sac MRCs, and those of the gills in juvenile and adult fish. Recent studies have also demonstrated the functional importance of these cells for Cl⁻ excretion in seawater (see Kaneko & Hiroi, this volume). It is conceivable and likely that skin and yolk-sac MRCs also play a vital role in acid-base regulation; however this remains to be investigated.

An in vitro "yolk ball" preparation has recently been developed (Shiraishi et al., 2001; see also kaneko & Hiroi, this volume), where the yolk-sac epithelium is separated from the developing larvae, and then incubated in a balanced salt solution so that the yolk-sac membrane, containing a rich population of MRCs, seals around the yolk completely. This preparation lends itself nicely to physiological studies to investigate the function of these MRCs in an environment that is very similar to in vivo conditions. To date, some studies have directly investigated the ionoregulatory but not acid-base regulatory role of MRCs using the "yolk ball" preparation.

Traditionally, the gills have been thought to develop for gas exchange in embryonic and larval fishes to compensate for a reduction in total surface area to volume that occurs with growth. However, this dogma has recently been challenged by Rombough (1999), who has proposed that the gills more likely develop for ionoregulation and/or acid-base regulation. This hypothesis is based upon numerous observations that MRCs develop on the gill lamellae long before the gills are required for gas exchange. For example, in rainbow trout (Oncorhynchus mykiss), MRCs appear on the gill filament a few days prior to hatch and long before the lamellae form. At hatch, the MRC density on the gill filament is already similar to that seen in adult fish (Perry et al., 1992), and the filament possesses 22% of larval MRCs despite the gills accounting for only 7% of total surface area (Rombough, 1999, 2004). Following yolk-sac absorption in rainbow trout, about 75% of the MRCs are found on the gills, while total gill surface area only accounts for 37% of the total body surface. There have been no studies conducted on the relative role of the gills, yolk-sac or skin surface for whole animal ionoregulation or acid-base regulation throughout early development and so this field remains a fruitful area for investigation.

In a SW teleost, the Japanese flounder, exposure to 1 and 5% CO₂ results in elevated gill NKA, and causes an increase in MRC size in larval Japanese sillage (Sillago japonica) and red seabream (Pagrus major), further supporting the role of the MRC in acid-base regulation during development in SW fishes (Ishimatsu et al., 2004). In FW, exposure to hypercapnia also affects MRC morphology as described above. Thus, MRCs are likely associated with acid-base regulation in larval fishes, at least during exposure to environmental hypercapnia, and changes in MRC characteristics are presumably indicative of processes associated with acid-base compensation during hypercapnia.

**ACID-BASE REGULATION IN RELATION TO THE ENVIRONMENT IN EARLY LIFE STAGES**

Many studies have investigated the effect of water pH on growth and survival of early life stages of fish (Eddy & Talbot, 1985; Alderice, 1988), largely in relation to concerns with acid rain and environmental acidification. Most of the negative effects of a low pH environment in juvenile and adult fish are thought to be more related to disruption of ionoregulation, than to acid-base disturbances per se (Wood, 1989). In some cases the ionoregulatory disturbances in juvenile and adult fish are associated with a change in gill permeability associated with low environmental pH; in others it is related to a limitation of the V-type H⁺-ATPase to generate an electrical gradient to drive Na⁺ uptake (Lin & Randall, 1991). In the latter case, compensation for the effect of low water pH may be informative on development of acid-base regulation for fish species that rely on net Na⁺/H⁺ exchange for acid-base regulation, but in general, these changes are more likely to be associated with adjustments to the ionoregulatory system.

Environmental hypercapnia is a useful tool to investigate the acid-base regulatory capacity as described above, but it is also of applied interest as global CO₂ levels continue to rise. Projected increases in atmospheric CO₂ levels are hypothesised to elevate surface water CO₂ levels and reduce the pH of these waters by up to 0.7 pH units (Caldeira & Wickett, 2003). CO₂ injection into the world’s oceans has been proposed as a means through which an elevation in atmospheric CO₂ levels may be minimised despite
continued industrial CO₂ production. The consequences of this practice on marine organisms have recently been questioned (Seibel & Walsh, 2001), as organisms will have to tolerate high local CO₂ concentrations at the site of disposal. While elevated CO₂ levels will reduce pH, there is more interest in CO₂ effects because of the high permeability of gaseous CO₂ relative to hydrogen ions. At constant levels of SW acidification, effects on embryos and larvae of marine fish were much greater if acidification was accomplished through hypercapnia than with acid addition (Kikikawa et al., 2004).

Recently, a number of studies have investigated acute CO₂ tolerance in marine fish species at different early developmental stages to address issues related to increases in global CO₂. The approach taken in these studies was more toxicological in design, where 6h and 24h LC₅₀ values (lethal concentration resulting in 50% mortality) could be calculated at different developmental stages. The basis for differences in CO₂ LC₅₀ values among species at similar developmental stages, or within species at different developmental stages, are likely related to: i) differences in tolerance to a given reduction in pH, ii) differences in buffer values that alter the magnitude of the acidosis at a given pCO₂, or iii) differences in active pH regulatory capacity up until the point that pH changes affect survival. From the time of fertilisation, through to larval and juvenile stages, there appears to be tremendous variability in CO₂ sensitivity, with 6h LC₅₀ ranging from 1 to 15% CO₂ (summarised in Ishimatsu et al., 2005) among species. There appear to be interesting patterns of CO₂ sensitivity with development. In two species of fish, Japanese silago and red seabream, the median lethal CO₂ at 6 h was between 1-3 kPa (1 and 3% CO₂) following cleavage, then increased to 6-7 kPa (6-7% CO₂) at the pre-flexion stage (Kikikawa et al., 2003). Following this, acute tolerance to CO₂ progressively decreased at the post-flexion and juvenile stage (3 kPa, 3% CO₂) to values similar to those of adult fish. The authors of this study attribute the reduction in acute CO₂ tolerance at the post-flexion and juvenile stages to gain development which initiates at the pre-flexion stage in these species. Perhaps the more intriguing question is what is responsible for the greatly elevated CO₂ tolerance at the pre-flexion stage, a time when great developmental changes are occurring, yet CO₂ sensitivity is dramatically reduced.

**FUTURE PERSPECTIVES**

Acid-base regulation is one of the most tightly controlled physiological processes in the body, due to the degree to which changes in pH can affect protein charge, enzyme function, metabolism and finally, whole animal performance. Most of the studies investigating the mechanisms and pathways involved in acid-base regulation in fish have been conducted in juvenile and adult life stages, and it is surprising how much remains unknown. In early life stages of fish, the ontogeny of acid-base regulation and mechanisms involved in acid-base regulation are vastly different from those in adults. Prior to hatching, fish develop a functional gill anatomy and proteinase systems, allowing them to adapt to their environment. Therefore, understanding the development of any system within the animal, and the influence that changes in the environment have on developmental trajectories is currently topical (Burggren & Warburton, 2005). Early life stages in fish are often considered the most sensitive to environmental perturbations, and the more we understand about the basic physiological mechanisms operating and their thresholds for perturbation the better able we are to make predictions about the organisms tolerance to changing environments, such as global changes in CO₂ levels. Investigations into the ontogeny of acid-base regulation in early life stages of fishes are sorely needed, and the possibilities endless. The following are particular areas in need of examination in relation to the material discussed above:

1. What is the relative role of the different surfaces (yolk-sac epithelium, body surface, gills) in acid-base regulation, and how do they change during development under different environmental conditions?
2. Are the same processes operating in developing fish as in juveniles and adults? For example, are some species, such as salmonids reliant upon net Na⁺/H⁺ exchange early in development, and then Cl⁻/HCO₃⁻ later in development?
3. What are the predominant acid-base regulatory mechanisms involved at the whole animal, tissue, cellular and protein level in developing fish?
4. Are MRC's the site of net acid/base exchanges between the developing fish and environment? How do changes in MRCs and expression of specific transporters correlate with whole animal performance during acid-base disturbances?
5. What are the implications of water hardness and salinity on rates of acid-base regulation in developing fishes?
6. What is the metabolic cost of acid-base regulation during development? Do elevated and/or fluctuating CO₂ levels associated with global warming or culture conditions influence growth and development?
7. Does ammonia accumulation in the whole body and yolk during development act as a temporal buffer system?

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