Physiological impacts of elevated carbon dioxide and ocean acidification on fish

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Heuer RM, Grosell M. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am J Physiol Regul Integr Comp Physiol 307: R1061–R1084, 2014. First published August 27, 2014; doi:10.1152/ajpregu.00064.2014.—Most fish studied to date efficiently compensate for a hypercapnic acid-base disturbance; however, many recent studies examining the effects of ocean acidification on fish have documented impacts at CO₂ levels predicted to occur before the end of this century. Notable impacts on neurosensory and behavioral endpoints, otolith growth, mitochondrial function, and metabolic rate demonstrate an unexpected sensitivity to current-day and near-future CO₂ levels. Most explanations for these effects seem to center on increases in PCO₂ and HCO₃⁻ that occur in the body during pH compensation for acid-base balance; however, few studies have measured these parameters at environmentally relevant CO₂ levels or directly related them to reported negative endpoints. This compensatory response is well documented, but noted variation in dynamic regulation of acid-base transport pathways across species, exposure levels, and exposure duration suggests that multiple strategies may be utilized to cope with hypercapnia. Understanding this regulation and changes in ion gradients in extracellular and intracellular compartments during CO₂ exposure could provide a basis for predicting sensitivity and explaining interspecies variation. Based on analysis of the existing literature, the present review presents a clear message that ocean acidification may cause significant effects on fish across multiple physiological systems, suggesting that pH compensation does not necessarily confer tolerance as downstream consequences and tradeoffs occur. It remains difficult to assess if acclimation responses during abrupt CO₂ exposures will translate to fitness impacts over longer timescales. Nonetheless, identifying mechanisms and processes that may be subject to selective pressure could be one of many important components of assessing adaptive capacity.

hypercapnia; CO₂; teleost; GABA; acid-base balance

AS ATMOSPHERIC CO₂ has increased from preindustrial (280 ppm CO₂) to present day values (~390 ppm CO₂) (66), equilibration with the ocean has led to a corresponding pH decline of 0.1 and is projected to undergo changes over the next two centuries at rates that have not been seen in the last 300 million years, ultimately leading to a pH decline of up to 0.77 by year 2300 (27, 146). Global mean oceanic CO₂ values are expected to reach 1,000 µAtm CO₂ by year 2100 and 1,900 µAtm CO₂ by year 2300 (27, 146), levels expected to impact a wide array of organisms (54, 128). Many valuable reviews have discussed the effects of ocean acidification on fish (117, 148, 162), and patterns of behavioral disturbance have been well characterized, especially in coral reef species (23, 135, 162). This review begins with an examination of ocean acidification studies over time and across taxa to explore the evolution and current status of fish studies in a broader context. Next, impacts of ocean acidification on fish are discussed with the intent of identifying common underlying physiological mechanisms and suggesting areas where future studies would be the most valuable. The review of primary literature forming the basis for the present review was completed January 15, 2014, although reference to a single and more recent primary paper on fish was added during the peer-review process.

Using a quantitative approach to enumerate the diversity and threshold levels of particular taxonomic groups can be an effective tool to identify patterns and determine critical knowledge gaps in the realm of ocean acidification research, as seen in previous meta-analyses (128, 129, 237). In this review, an examination of peer-reviewed literature from 2000 to 2013 revealed that research across most taxa began to rapidly expand after 2007 (Fig. 1). The calcifying Cnidaria and Molluscs are the most studied taxonomic groups and have seen the most rapid increase in the number of studies per year, averaging ~52–57 studies per year since 2012 (Fig. 1). The low number of studies on marine worms and sponges stands in stark contrast to the aforementioned groups with ~5–8 studies per year from 2012–2013, suggesting research on these less prominent but ecologically important groups is needed (Fig. 1). Pisces, Echinoderms, and Crustaceans are at an intermediate level (~27–30 studies/year). Before ~2009, studies examining...
CO2 effects on fish groups were performed at relatively high levels and were largely mechanistic in nature, demonstrating that fish are effective acid-base regulators (see Acid-Base Balance) (Fig. 1). This regulatory ability, combined with early studies showing effects on growth and survival only at high CO2 exposures, undoubtedly led to the idea that fish were not particularly sensitive to CO2. Broadening the scope of tested endpoints revealed that fish are far less tolerant to low CO2 levels (less than ~2,000 µAtm CO2) than predicted, a shift largely prompted by the olfactory disturbance noted in the orange clownfish at 1,000/µAtm CO2; however, studies utilizing higher CO2 levels will also be discussed to provide context for acid-base balance. However, few studies have quantified the time course, threshold, effects of long-term chronic exposure, and magnitude of change in acid-base chemistry that occur in extracellular and intracellular fluids in fish during low level CO2 exposure. These types of physiological measurements, in addition to molecular and biochemical techniques, are of central importance to assessing the impacts of ocean acidification on fish populations, especially since there is likely substantial variation across species, life stages, and environments. A more mechanistic understanding of how CO2 affects fish could serve in a predictive capacity for other measured physiological pathways and would likely be informative in the context of synergistic stressors such as hypoxia and temperature (188). While the idea that changes in acid-base balance to cope with future projected ocean acidification may present fish with a need for physiological tradeoffs and metabolic costs is certainly not new (117, 148, 190), the rapid increase in studies examining how CO2 affects fish merits a review of studies performed to date. Ocean acidification-relevant CO2 levels are generally considered to fall under ~2,000 µAtm CO2; however, studies utilizing higher CO2 levels will also be discussed to provide context and/or stimulate hypotheses that could be relevant at lower CO2 levels. Furthermore, it is important to note that CO2 impacts on freshwater fish are occasionally referenced but may not be directly applicable to marine fish due to differences in osmoregulatory and acid-base balance strategies (see Acid-Base Balance).

Environmental and Adaptive Considerations

One important, but largely unavoidable caveat to most fish ocean acidification studies is that abrupt and relatively short-term CO2 exposures are inherently limiting in their ability to assess the adaptive capacity of a species. Despite this constraint, research on impacted mechanisms and pathways may
show which traits are most susceptible to selective pressure (124). Multigenerational studies confined to species with shorter life spans and/or assessment of standing variation are needed to more directly assess the scope for adaptation (124). One study examining fish living near natural highly acidic CO2 seeps found behavioral defects similar to those noted in current (154). Some effects are tent across generations (154). Limited fish studies have aimed to quantify epigenetic effects with mixed results. Some effects are needed to more directly assess the scope for adaptation (124). Individual publications may be included in more than one effect subcategory; however, each data point represents the lowest tested effect level in one subcategory for a particular species. Accordingly, studies examining multiple species could be counted multiple times across subcategories. Species examined in multiple studies were only reported once at the lowest effect level in a subcategory. It is important to note that the only subcategory (ventilation) effect reported in the Cardiorespiratory category is from a freshwater species (see text). See online supplemental Table 1 for more detail and literature references.
a stressor may facilitate a more targeted approach in assessing the adaptive capacity of a species (210).

Although our analysis of CO₂ effects on fish largely focuses on exploring the physiology underlying significant effects, around half of all tested parameters show no effect or even a seemingly positive effect (online supplemental Table 1). This variation makes it difficult to assess the true level of sensitivity of fish to ocean acidification as a taxon. Authors of a recent review (237) suggest that more studies are needed to confirm the recently observed high sensitivity in fish, since fish from previous high CO₂ paleo-events appear less sensitive.

**Acid-Base Balance**

Depending on the level, elevated ambient CO₂ either reduces or reverses the PCO₂ gradient from aquatic animals to their environment until a new steady state is reached (119), acting to reduce or eliminate CO₂ excretion, respectively. In either case, internal PCO₂ levels will increase and may result in acidification of internal fluid compartments unless compensation occurs.

**Extracellular pH regulation.** Teleost fish maintain relatively constant, yet temperature-dependent, alkaline pH (7.7–8.1) in extracellular fluids (pHₑ) (142). Owing to the largely O₂-based ventilatory drive, the relatively high CO₂ solubility in water, the unidirectional water flow across respiratory surfaces, and the countercurrent exchange of water and blood flow at the gill, CO₂ is readily excreted into the water resulting in low plasma PCO₂ in strict water breathers compared with air-breathing vertebrates (arterial PCO₂ ~2 Torr or ~2,600 μAtm in fish vs. ~40 Torr or ~53,000 μAtm in humans) (37, 60, 105, 180). Respiratory adjustments to control acid-base balance are thus relatively inefficient strategies in water-breathing compared with air-breathing animals due to the low scope for PCO₂ regulation (60, 180), although cardiorespiratory responses by fish exposed to elevated CO₂ have been noted (see Cardiorespiratory Responses to Elevated CO₂). Rather, the main response of fish to acid-base balance disturbance is to adjust blood plasma HCO₃⁻ levels through the differential regulation of H⁺ and HCO₃⁻ excretion rates (metabolic adjustment) (28, 60, 142, 178, 180, 193).

Historically, elevated CO₂ (hypercapnia) has been frequently used in studies of acid-base balance to induce a respiratory acidosis in freshwater and marine fish (30, 100, 119, 137, 192). Most studies demonstrate full and efficient pH compensation within hours to days of initial acidosis despite continued exposure to hypercapnia at levels exceeding 10,000 μAtm, a process that is achieved by retention and/or uptake of extracellular HCO₃⁻ and/or by an increased net acid secretion (28, 29, 39, 60, 88, 106, 107, 134, 142, 144, 150, 176, 180, 181, 193, 224, 232, 240). While these observations clearly illustrate fish are highly efficient acid-base regulators, they have undoubtedly led to the general assumption that compensation confers broad CO₂ tolerance (98, 129, 148, 188). Recent research noting sublethal CO₂ effects as low as ~500 μAtm CO₂ (online supplemental Table 1) and frequently under 1,000 (Fig. 2), suggests that while this compensation protects extracellular pH status, there may be other downstream consequences or tradeoffs. The first study to investigate acid-base balance responses of a subtropical fish to more environmentally realistic PCO₂ levels (560–1,900 μAtm) revealed a similar pattern of initial respiratory acidosis followed by metabolic compensation (≥750 μAtm) within a few hours in the form of elevated plasma HCO₃⁻ (58). An Antarctic fish exposed to 2,000 μAtm has also shown complete protection of pHₑ by elevation of plasma HCO₃⁻, although the time required for this compensation at lower temperatures remains to be established (215). Thus fish exposed to elevated CO₂ effectively regulate extracellular pH but display continuously elevated PCO₂ and HCO₃⁻ in their extracellular fluids. While pHₑ is normalized, the elevated PCO₂ and HCO₃⁻ concentrations are presumed to have pronounced implications for behavior (see Neurosensory and Behavior), otolith growth (see Calcification and Osmoregulation) even at relatively low ambient PCO₂ levels.

**Intracellular pH regulation.** Even modest decreases in intracellular pH (pHi) can result in reductions of glycolysis and impairment of aerobic metabolism (103, 211). Consequently, it is not surprising that pHₑ is defended and tightly regulated in most animals (239). The regulation of pHₑ is likely attributable, in part, to Cl⁻/HCO₃⁻ exchange (18, 42, 199, 214) or acid extrusion via Na⁺/H⁺ exchange (39, 130, 168, 170, 199) in most tissues and also to catecholamine activated Na⁺/H⁺ exchange in fish red blood cells (RBCs) (8, 12, 20). What appears to be characteristic for pHₑ regulation in fish during exposure to moderately elevated PCO₂ is not only a lack of pHi acidification, but in some cases, a pHₑ overshoot associated with pHₑ compensation. Such an overshoot has been reported for white muscle of the subtropical gulf toadfish exposed to 1,900 μAtm for 24 h (~ΔpH 0.07) (58) and for RBCs, brain, liver, and heart of the temperate white sturgeon exposed to 15,000 μAtm (8) for 48 h. In contrast, white muscle and liver of the Antarctic marbled rockcod displayed pHₑ control during exposure to 2,000 μAtm (215). Regardless of the tissue examined, all three species show a regulation or overshoot of pHₑ and a PCO₂ increase, combined with a presumed (58) or calculated (8, 215) substantial accumulation of intracellular HCO₃⁻ (~ΔHCO₃⁻ 1–3 mM in rockcod (215)) and/or by an increased net acid secretion by fish exposed to elevated CO₂ have been noted (see Cardiorespiratory Responses to Elevated CO₂). Rather, the main response of fish to acid-base balance disturbance is to adjust blood plasma HCO₃⁻ levels through the differential regulation of H⁺ and HCO₃⁻ excretion rates (metabolic adjustment) (28, 60, 142, 178, 180, 193).

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**Acid-base regulation organs.** The gill is the primary acid-base regulatory organ in teleosts, and it appears that reductions in pHi under climate change PCO₂ are not only a lack of pHi acidification, but in some cases, a pHₑ overshoot associated with pHₑ compensation. Such an overshoot has been reported for white muscle of the subtropical gulf toadfish exposed to 1,900 μAtm for 24 h (~ΔpH 0.07) (58) and for RBCs, brain, liver, and heart of the temperate white sturgeon exposed to 15,000 μAtm (8) for 48 h. In contrast, white muscle and liver of the Antarctic marbled rockcod displayed pHₑ control during exposure to 2,000 μAtm (215). Regarding of the tissue examined, all three species show a regulation or overshoot of pHₑ and a PCO₂ increase, combined with a presumed (58) or calculated (8, 215) substantial accumulation of intracellular HCO₃⁻ (~ΔHCO₃⁻ 1–3 mM in sturgeon (8) and ~ΔHCO₃⁻ 1–3 mM in rockcod (215)) and/or by an increased net acid secretion by fish exposed to elevated CO₂ have been noted (see Cardiorespiratory Responses to Elevated CO₂). Rather, the main response of fish to acid-base balance disturbance is to adjust blood plasma HCO₃⁻ levels through the differential regulation of H⁺ and HCO₃⁻ excretion rates (metabolic adjustment) (28, 60, 142, 178, 180, 193).

Although the gill is the main organ for acid-base exchange in freshwater fish (29, 60, 107, 144, 232, 240), the kidney plays a significant role by reabsorbing HCO₃⁻ uptake across the gill epithelium (58, 106), implicating H⁺ as well as HCO₃⁻ transporters (Fig. 3). While the outcome of these two mechanisms is the same for the fish in terms of acid-base balance (a net acid loss), mechanistic insight into compensation for respiratory acidosis will aid interpretations of responses to elevated PCO₂.

In contrast to freshwater fish, the renal contribution to marine fish acid-base balance is of modest importance (38–40, 46) owing, in part, to the much lower urine flow rates (~0.3 ml·kg⁻¹·h⁻¹) than in freshwater teleosts (~3.0 ml·kg⁻¹·h⁻¹) (142). Furthermore, it appears that urine pH or urine flow of the marine gulf toadfish are not influenced by hypersalinity (70 ppt) (81), a treatment that results in a doubling of branchial acid excretion.
Unlike freshwater fish, marine teleosts secrete base into the intestinal lumen, via \( \text{Cl}^-/\text{HCO}_3^- \) exchange, resulting in high rates of rectal base excretion (89, 90, 94) (see *Calcification*). This intestinal base secretion serves osmoregulatory purposes and varies with ambient salinity (82), time after feeding (222), and was recently documented to respond to low ambient \( \text{CO}_2 \) (red). The salt secretory pathway of the marine teleost intestine consists of basolateral entry of \( \text{Na}^+ \), \( \text{K}^+ \), and \( \text{Cl}^- \) via \( \text{Na}^+/-\text{K}^+/-\text{Cl}^- \) cotransporter (NKCC1) driven by \( \text{Na}^+/-\text{K}^+/-\text{ATPase} \) activity (NKA). Secretion of \( \text{Cl}^- \) is transcellular via NKCC1 and apical cystic fibrosis transmembrane conductance regulator (CFTR) while \( \text{Na}^+ \) exits via a paracellular pathway down its electrochemical gradient and \( \text{K}^+ \) recycles across the basolateral membrane via a \( \text{K}^+ \) channel (not shown). Hydration of \( \text{CO}_2 \), facilitated by carbonic anhydrase (CAc), generates \( \text{H}^+ \) and \( \text{HCO}_3^- \) for exchange and cotransport across the apical and basolateral membranes. It should be noted that a number of CA isoforms, in addition to CAc, likely are present and contribute to \( \text{CO}_2 \) hydration in the gill tissue. At least two \( \text{Na}^+/-\text{H}^+ \) exchanger isoforms (NHE3 and NHE1) and likely a third (NHE2) are expressed in the gill epithelium, and it appears that both the apical NHE3 and the basolateral NHE1 are involved in dynamic adjustments of acid-base balance during hypercapnia-induced acidosis. The upregulation of NHE3 and the downregulation of NHE1 as well as the basolateral proton pump is consistent with a need for net acid excretion during exposure to elevated \( \text{CO}_2 \). To date, none of the three \( \text{HCO}_3^- \) transporters from the SLC26 family (A3, A4, and A6), have been demonstrated to contribute to the dynamic acid-base balance regulation of marine fish exposed to elevated \( \text{CO}_2 \), while the apical SLC4a2 member AE2 and the basolateral A4 members SLC4a1 (AE1) and SLC4a4 (NBC1) both respond to \( \text{CO}_2 \) exposure in a manner that appears adaptive. Considering the role of \( \text{Na}^+ \) and \( \text{Cl}^- \) balances in transport of acid and base equivalents, it is not surprising that most studies report an elevation of NKA expression and/or activity. See text for further detail.

**Cellular transport mechanisms.** The impressive acid-base regulatory capacity of fish is generally credited to the concurrent transport pathways for \( \text{H}^+ \) and \( \text{HCO}_3^- \) across the basolateral and apical membranes of the gill epithelium (Fig. 3). Although changes to these transport mechanisms are often evaluated by examining changes in mRNA expression or enzymatic activity, other strategies, such as regulation at the protein level by phosphorylation and protein trafficking, may also be employed.

**H\(^+\) transport.** First, considering gill \( \text{H}^+ \)-transporters, at least three \( \text{Na}^+/-\text{H}^+ \) exchanger isoforms (NHE1, NHE2, and NHE3) and the vacuolar \( \text{H}^+ \) pump are relevant for fish acid-base balance. Elegant studies by Claiborne and colleagues (38) first implicated the basolateral NHE1 isoform in compensation for metabolic acidosis. Since then, this NHE isoform (NHE1a and NHE1b) has been implicated in acid-base balance regulation during hypercapnia-induced respiratory acidosis in other studies at relatively high \( \text{PcO}_2 \) levels (10,000–52,000 \( \mu \text{Atm} \)) (46, 56, 195). Downregulation of NHE1 mRNA expression is common to all studies reporting effects of hypercapnia, which is consistent with a need for reduced transport of \( \text{H}^+ \) by this basolateral isoform from the gill epithelium to the blood plasma (Fig. 3). Of the two apical NHE isoforms, NHE3 has been reported to show elevated gill protein abundance following exposure to 10,000 \( \mu \text{Atm} \) (56), and elevated mRNA expression of NHE3 has been reported for early-life stages of fish exposed to levels as low as 1,200 \( \mu \text{Atm} \) (229). The increase in mRNA expression and protein abundance for the apical NHE3 is consistent with a need for net acid excretion during exposure to hypercapnia. At present there is no information suggesting involvement of NHE2 in marine fish gill responses to hypercapnia-induced acidosis. An early report demonstrated consistent reductions in gill \( \text{H}^+ \) pump (V-type
exposed to 10,000 and 1,200–7,000 μAm, respectively. After an initial and transient depression, NBC1 mRNA expression was found to be elevated in eelpout after 42 days of exposure to 10,000 μAm, consistent with a need for transport of HCO₃⁻ from the gill epithelium across the basolateral membrane (46).

In agreement with these observations are reports of elevated whole body mRNA expression of the NBC1a isoform in newly hatched medaka exposed to ≥1,200 μAm and of NBC1b in the gill tissue of adult medaka exposed to 7,000 μAm (229). Interestingly, the NBC1a isoform in adult medaka gills at 7,000 μAm and embryos at 1,200 and 4,200 μAm displayed a suppressed mRNA expression. The NBC1b isoform was also suppressed in embryos at 4,200 μAm; however, this isoform showed no mRNA expression changes in embryos at 1,200 μAm or newly hatched larvae at 1,200 and 4,200 μAm (229).

The diversity of NBC1 regulatory responses suggests that multiple strategies in adjusting cellular machinery may be elicited during CO₂ exposure, emphasizing the critical need for further research detailing cellular responses spanning species, exposure time, and CO₂ levels.

Carbonic anhydrase. The acid-base regulatory adeptness of fish hinges in part on the hydration of CO₂ within the gill epithelium, which provides H⁺ and HCO₃⁻ for differentially regulated apical as well as basolateral extrusion to the water and blood, respectively (Fig. 3). The cytosolic CO₂ hydration is catalyzed by carbonic anhydrase (CA) isoforms of which the cytosolic CAc (similar to mammalian CAII) is the best characterized (59, 83, 204). This CA isoform displays increased enzymatic activity in response to salinity (60 ppt) elevated above ambient, a challenge that elicits increased net branchial acid excretion. Considering the pivotal role of this group of enzymes in gill acid-base and ionicregulatory functions, it is surprising that relatively little attention has been directed to the impact of elevated CO₂ on CA isoforms in the gills and other tissues. However, it is not surprising that CA responses to CO₂ exposure have been both clear and consistent in the two studies performed to date at environmentally relevant Pco₂ levels (58, 229). Gill CAc mRNA in the gulf toadfish exposed to 1,900 μAm for 8–72 h exhibited a strong downregulation (58), which has also been demonstrated in embryos and larvae of medaka exposed to >1,200 and 4,200 μAm, respectively (229). In addition to reports of effects of elevated Pco₂ on CAc, another isoform, CA15 mRNA, is downregulated in response to elevated Pco₂ at least in medaka embryos (229). Although one study employing very high Pco₂ levels (50,000 μAm) reported elevated CA protein abundance and enzymatic activity in gill tissue (178), the downregulation seen at lower Pco₂ levels supports the observation that the compensatory response to elevated Pco₂ involves HCO₃⁻ uptake rather than H⁺ excretion (58). CA within the gill tissue would not benefit uptake of ambient HCO₃⁻, since an elevation of cytosolic HCO₃⁻ could make uptake from seawater less favorable. The elevation of gill CA at higher Pco₂ levels may reflect that more extreme conditions require H⁺ excretion, and thus gill CA, to defend plasma pH. The study of Pco₂-induced modifications of CA isoform functions in fish gill tissue is clearly a fruitful area for further studies.

\[ \text{Na}^+\text{-K}^+\text{-ATPase} \]

While a single study has demonstrated a transient downregulation in enzymatic activity (1,900 μAm CO₂) (58), a number of studies report elevated expression of NKA subunits and/or NKA activity in gill tissues of fish...
exposed to elevated PCO2 [6,000 μAtm (147), 10,000 μAtm (46)]. While such elevations may correspond to a need for compensation for the increased salt absorption by the intestine of CO2-exposed fish (111) (see Osmoregulation), it may also reflect the need for increased activity of acid and base transporters relying on Na+ and Cl− gradients. These transporters include the NHEs, anion exchangers, and cotransporters discussed above.

Rh proteins. Although not involved directly in transport of H+ or HCO3−, branchial Rh proteins may contribute to acid-base regulation as they transport ammonia (NH3) across the gill epithelium for excretion. Some evidence also suggests these proteins are capable of transporting CO2 in certain cell types (165, 179). The apical Rhcg isoform operates in concert with NHE3 to excrete NH3 and H+, respectively, in seawater-acclimated puffer fish leading to NH2− formation at the boundary layer of the apical gill surface, facilitating continued diffusion of NH3 (164). Although this process does not contribute to net transfer of acid-base equivalents, it is nevertheless interesting that a number of Rh mRNAs (Rhag, Rhbg, Rhcg) have been reported to respond to elevated PCO2 (1,200 – 7,000 μAtm) in medaka embryos (increase and decrease), larvae (increase), and gills of adults (decrease and increase) (229). Whether these expression changes reflect a direct role of Rh proteins in acid-base balance regulation or whether they represent changes in conditions for ammonia excretion dictated by the elevated PCO2 remains to be investigated.

Calcification

Fish are not traditionally considered calcifying organisms even though they produce CaCO3 precipitates in the intestinal lumen and otoliths in the inner ear. Otoliths are CaCO3 concretions that function in sound detection and as gravity-sensing organs (225, 226), whereas intestinal CaCO3 precipitates are produced to serve intestinal water absorption and thus osmoregulation (89, 90, 92, 235).

Otoliths increase in size and density during exposure to elevated CO2. A study on sea bass refuted the hypothesis that reduced aragonite saturation states in water with elevated CO2 would impair otolith growth and demonstrated increased rather than decreased otolith size in larval fish exposed to environmentally realistic CO2 levels of 993 μAtm (34). Similar observations have since been reported for clownfish exposed to 1,721 μAtm (160), cobia exposed to 800 μAtm (14), Atlantic cod exposed to 1,800 μAtm (141), and walleye pollock exposed to 478 μAtm (115). In addition, increased otolith mass has been documented from cobia exposed to 800 μAtm (13). However, no effect of elevated CO2 on otolith growth has also been reported for the spiny damselfish exposed to levels up to 900 μAtm (159, 209), eastern Baltic cod exposed up to 4,000 μAtm (80), and Atlantic herring exposed to levels as high as 4,635 μAtm (76), illustrating species-specific difference in susceptibility to CO2-induced alterations in otolith growth. Regardless of these differences among species, observations of increased otolith growth likely reflect the elevated plasma Pco2 and HCO3− levels observed in at least some marine fish exposed to CO2 (see Acid-Base Balance), as both likely serve as substrate for carbonate aggregation in otoliths. Otoliths are confined within a saccular epithelium containing alkaline endolymph enriched in total CO2 with concentrations up to 32 mM (172, 173), 5–10 times greater than blood plasma values. Incorporation of H14CO3− into endolymph as well as otoliths display saturation kinetics in vitro when H14CO3− concentrations in the bathing medium outside the epithelium are increased within physiological blood plasma HCO3− concentrations (226). These observations demonstrated carrier-mediated HCO3− import from the blood to the endolymph for otolith formation (Fig. 4) and may, at least in part, account for observations of increased otolith size when fish compensate for CO2 exposure by elevating plasma HCO3− concentrations. Indeed, carbonate incorporation into endolymph and otoliths increase with increasing HCO3− concentrations in the bathing solutions and saturates at about 25 and 10 mM, respectively (226). Thus increases in plasma HCO3− levels of ~1 and 3 mM seen in toadfish exposed to 1,000 and 1,900 μAtm, respectively (58), can be expected to result in increased carbonate incorporation into otoliths. Thus plasma HCO3− increases reported from fish exposed to environmentally realistic CO2 levels are of sufficient magnitude to increase CO32− incorporation into otoliths.

While fish effectively compensate and correct for a reduction in pH during CO2 exposure by increasing plasma HCO3−, plasma Pco2 remains elevated (58). In addition to blood plasma HCO3−, hydration of CO2 within the saccular epithelium likely
provides $\text{HCO}_3^-$ as substrate for otolith formation. Observations of high CA mediated CO2 hydration rates in the cytosolic fraction of the saccular epithelium as well as immunoreactivity with a CAII antibody illustrate the presence and function of this enzyme (225). Application of the CA inhibitor acetazolamide to isolated sacculi results in substantial inhibition of $\text{CO}_2^-$ incorporation into endolymph as well as otoliths (226), demonstrating a role for cytosolic CA in hydration of endogenous CO2 to provide substrate for otolith formation. It seems reasonable to assume that elevated extracellular CO2 would result in elevated intracellular CO2, which could provide cellular HCO$_3^-$ via CAII hydration in the saccular epithelium for transport into the endolymph and incorporation into otoliths (Fig. 4). However, a relationship between extracellular CO2 and secretion of HCO$_3^-$ by the saccular epithelium and incorporation into otoliths remains to be established. Furthermore, with two exceptions (58, 215), blood acid-base status has not been monitored in fish exposed to realistic CO2 levels, and it is therefore unclear if the observed species difference in response to CO2 with respect to otolith growth reflects differences in blood acid-base status or other physiological traits.

**Intestinal CaCO$_3$ formation.** Marine fish rely on seawater ingestion and solute-coupled water absorption by the intestine to maintain water balance (see Osmoregulation) (92). While cotransporters Na$^+$/K$^+$/2Cl$^-$ (NKCC2) and Na$^+$/Cl$^-$ (NCC) contribute to absorption of Na$^+$ and Cl$^-$, Cl$^-$/HCO$_3^-$ anion exchange accounts for up to 70% of intestinal Cl$^-$ uptake (89) (Fig. 5). One consequence of this substantial anion exchange is very high luminal HCO$_3^-$ concentrations (up to 100 mM) and alkaline conditions (pH in some cases exceeding 9.0), which result in precipitation reactions with Ca$^{2+}$ from ingested seawater to form macroscopic CaCO$_3$ aggregates within the intestinal fluids (235). The CaCO$_3$ formation reduces luminal osmotic pressure as much as 100 mosM and thereby facilitates osmotic fluid absorption by the intestine (5, 90, 91, 235). Indeed, recent studies revealed that the reduction in luminal osmotic pressure arising from the CaCO$_3$ precipitate reaction is critical for successful osmoregulation and thus survival of marine fish (81).

Transepithelial HCO$_3^-$ transport from the blood to the intestinal lumen, as well as hydration of endogenous CO2 arising from the high metabolic rates of the intestinal epithelium (222), provide two sources of HCO$_3^-$ for secretion into the intestinal lumen. With few exceptions (94), these two sources contribute about equally to overall intestinal HCO$_3^-$ secretion (93, 233). At least for the species examined so far, transepithelial HCO$_3^-$ transport is mediated by an electroneutral basolateral Na$^+/\text{HCO}_3^-$ cotransporter (SLC4a4) (33, 131, 221) and an electrogenic apical Cl$^-$/HCO$_3^-$ exchanger (SLC26a6) (96, 131) (Fig. 5) and appears to be limited mainly by the basolateral step (221). Hydration of endogenous CO2 arising from the metabolic activity of the active intestinal epithelium is catalyzed by cytosolic CA (CAc; equivalent to mammalian CAII). This hydration reaction provides HCO$_3^-$ for apical anion exchange via SLC26a6 and H$^+$ for secretion mainly across the basolateral membrane via Na$^+$-dependent pathway, likely NHE1 (93–95, 204) (Fig. 5).

Intestinal HCO$_3^-$ secretion by marine fish is stimulated both by elevated extracellular, serumal CO2 (97) and by elevated serosal HCO$_3^-$ (93, 97, 221, 233). Thus the compensatory response to elevated ambient CO2 resulting in increased plasma Pco2 and increased plasma HCO$_3^-$ (see Acid-Base Balance) could be hypothesized to stimulate intestinal HCO$_3^-$ secretion. Indeed, this hypothesis was first confirmed for midshipmen exposed to highly elevated ambient CO2 (50,000 μAtm) (178) and has since been demonstrated for toadfish exposed to environmentally realistic CO2 levels (1,900 μAtm) (111).

Intestinal CaCO$_3$ precipitation and elimination to the environment has been shown to reflect overall intestinal HCO$_3^-$ secretion (82). Thus predictions of elevated CaCO$_3$ release by fish exposed to elevated CO2 seem reasonable and are interesting in the context of ocean acidification as it would represent an unusual scenario of elevated calcification and CaCO$_3$ production in response to reduced ambient aragonite saturation. Notably, fish-produced CaCO$_3$ contributes significantly to the oceanic inorganic carbon cycle and sedimentation of CaCO$_3$ (175, 203, 234). Such elevated CaCO$_3$ excretion was observed for midshipmen exposed to 50,000 μAtm (178) (four- to fivefold). In contrast, a study on toadfish using environmentally realistic CO2 levels of 1,900 μAtm found no significant increase in CaCO$_3$ production despite an overall significant increase in intestinal HCO$_3^-$ secretion of 33% (111). The fact that this endpoint has been examined in what are commonly perceived as “tolerant” species, combined with the large disparity in test levels (×25-fold), suggests that CaCO$_3$ formation is understudied with respect to hypercapnia and the possibility of increased precipitation in other species should be examined. Even if changes in CaCO$_3$ production are minimal under predicted ocean acidification, increased overall base excretion is counterproductive under elevated CO2 conditions where HCO$_3^-$ retention is required to maintain blood pH (111), creating a situation that may require adjustments in the intestinal tissue and/or the gill. Possible acclimation responses, tradeoffs, and metabolic costs of intestinal HCO$_3^-$ secretion and potential interactions with osmoregulatory processes during prolonged CO2 exposure is an area in need of attention.

As mentioned above, fish CaCO$_3$ contributes significantly to global oceanic CaCO$_3^-$ production (175, 203, 234). Even though the impact of elevated ambient CO2 on fish CaCO$_3$ excretion rates could be modest at environmentally realistic CO2 levels, it may nevertheless be of importance to understanding carbonate fluxes in oceans under future CO2 scenarios. Relatively few species, mainly sedentary and benthic, have been examined for CaCO$_3$ excretion rates, and the impacts of CO2 have only been tested in two species, pointing to a need for additional studies. This point is underscored by recent reports of relatively high solubility of the Mg$^{2+}$-rich fish-produced CaCO$_3$ (241), although this claim is currently being challenged (63, 73). Furthermore, substantial variations among species in the Mg$^{2+}$ content of fish produced CaCO$_3$ (203) suggests that solubility of these CaCO$_3$ and thus their fate with respect to sedimentation and/or dissolution may vary among species.

**Neurosensory and Behavior**

The most extensive body of research examining the effects of ocean acidification on fish has focused on sensory systems and behavior. Robust and consistent disturbances have been noted across a range of sensory systems including olfaction (44, 49–52, 156, 157, 163), hearing (209), and vision (70, 74).
and has also been implicated in processes linked to general cognitive function including undesirable changes in lateralization (53, 121), activity (44, 51, 68, 157, 163), boldness (68, 157, 163), and learning (36, 69, 121). With the exception of one recent study illustrating retained olfactory ability in Atlantic cod at 1,000 μAtm (122), these impairments have been noted in species at numerous life stages and in both tropical and temperate species. Most importantly, almost all lowest pCO2 exposures that induced significant effects in this topic area (online supplemental Table 1, Fig. 2) have been reported below IPCC models for the end of this century (146), providing evidence that fish are far less tolerant to ocean acidification than previously predicted during acute exposures. In fact, at least three studies to date have directly linked lab-demonstrated sensory disruptions to increased mortality in the field (36, 68, 157). The implications of these disturbances are expected to be substantial and include changes to dispersal (156), recruitment (68), connectivity (156), social interactions (51), predator-prey dynamics (2, 44, 68, 70, 71, 138, 163), population replenishment (52, 157), biodiversity (156, 157), habitat preference (49, 50), and settlement timing (49); all of which could drastically affect population and ecosystem dynamics (see Refs. 23 and 162 for reviews). While most studies point to decreased sensory ability, authors of a recent study showing increased...
phototaxic response in goby larvae suggest that this presumed increase in visual ability is likely due to an undesireable overstimulation rather than an enhancement (74). A central theme, which will serve as the focus for the rest of this section, is to discuss the possible underlying physiological mechanisms responsible for these disturbances. While some work on this topic is emerging (3, 36, 58, 99, 166), more studies are needed to fully understand the consequences induced during a compensated CO₂-induced acidosis (see Acid-base Balance).

As mentioned in other studies and reviews, disruption to endpoints representing broad cognitive impairment such as lateralization and learning suggest that ocean acidification affects central neural processing rather than individual sensory systems in isolation (49, 53, 69, 70, 166, 236). Disruption of multiple sensory systems in a single damselfish species (Pomacentrus amboinensis) (68–70, 138) and in the orange clownfish (Amphiprion percula) (157, 209) further support this idea. Interestingly, the minimum CO₂ level needed to invoke these responses varies, which could reflect differences in processing of signals from each sensory system and/or how these signals are integrated into neuronal systems (70). Changes in sensory system structures have been investigated but have generally not been linked to behavior, further supporting broader neural disruptions as the cause of behavioral changes (156). For example, olfactory disturbances were not found to be linked with structural changes in the nasal area (156), and increases in otolith size have not been convincingly linked to behavioral endpoints. In clownfish, auditory disturbance occurs at a much lower CO₂ threshold (600 μAtm CO₂) (209) than what is observed for increases in otolith growth (1,700 μAtm CO₂) (160), and cobia displaying increased otolith growth (800 μAtm CO₂) show only minor changes in swimming activity (14). Evidence for centralized processing impacts is also present in studies where fish prefer an inappropriate scent over control water (52, 156, 157, 163), show a lack of preference when one should be present (49–51, 158, 209), or a switch from preference to avoidance (44, 49). While the input, processing, and integration of sensory signals in the brain occur rapidly, sensory and behavioral responses in CO₂ exposed fish typically take ~4 days to manifest (157), are elicited in settlement stage larvae but not young hatchlings exposed to elevated CO₂ from the embryonic stage (52), and are reversible after ~2 or 12 days once fish are returned to control water (tropical damselfish and temperate Californian rockfish, respectively) (99, 157). As previously suggested, these findings suggest that contact of sensory structures with seawater of altered PCO₂ does not instantly change input but modifies responses on a longer timescale that could involve regulation of neuronal processes related to acid-base balance compensation (see Acid-Base Balance) (36, 44, 53, 69, 70, 121, 156, 157, 166, 209). Few studies have discussed specific neuronal cells/regions (2, 3, 99) or brain hemisphere communication errors (53) that could be linked to specific observed behavioral endpoints. Interestingly, a recent study has demonstrated that certain neurological disruptions, represented by a set of locomotor and nonlocomotor aspects of escape time, may be attenuated in juveniles when parents are also exposed to CO₂ (3).

An important stride in understanding the basis for neurosensory disruptions in fish was reported by Nilsson and colleagues in 2012 (166), who demonstrated that the primary background inhibitory neurotransmitter GABA and its accompanying GABA_A receptors in the nervous system were associated with disruptions in olfaction and lateralization during CO₂ exposure (166). In this study, the addition of gabazine, a GABA_A receptor antagonist, was found to reestablish proper olfactory function and lateralization in CO₂-exposed fish. It has been suggested that decreases in plasma Cl⁻ and increases in HCO₃⁻ during compensation for a CO₂-induced acidosis (see Acid-Base Balance) alter normal neuronal cell membrane ion gradients leading to an efﬂux rather than an inﬂux of these ions during GABA binding to GABA_A receptors. This change from membrane hyperpolarization to depolarization leads to an excitatory rather than an inhibitory response, thus providing an explanation for the alleviation of CO₂ effects with gabazine addition (36, 166). Recently, two subsequent studies have reinforced GABA_A involvement in CO₂-related sensory impairment related to learning (36) and anxiety (99). In the former study, CO₂-exposed fish taught to recognize a predator cue in the presence of gabazine were not able to respond to this cue 1 day after the exercise due to lasting CO₂ effects but were able to successfully respond 5 days later when CO₂ effects would have ceased, demonstrating that fish could receive sensory information during CO₂ exposure but could not properly process the signals. (36). In the latter study, CO₂ fish treated with muscimol, a GABA_A receptor agonist, exhibited increased anxiety levels, while control fish treated with the same drug exhibited reduced anxiety (99). These divergent responses using a drug designed to open the GABA_A receptor effectively demonstrated that changes within the body during CO₂ exposure are capable of inducing a depolarizing (excitatory) rather than a hyperpolarizing (inhibitory) current (99). The GABA_A receptor exhibits conductance for both HCO₃⁻ and Cl⁻ and could therefore be affected by changes in gradients of these ions due to elevated CO₂. Although both HCO₃⁻ and Cl⁻ mediate current across GABA_A receptors, there are variable measures of GABA_A receptors’ relative HCO₃⁻:Cl⁻ permeability in neurons from different species including invertebrates and mammals ranging from ~0.2 to 0.6 (64), and it is generally assumed that Cl⁻ is more permeable (133). The pharmaceutical compounds utilized in fish ocean acidification experiments, gabazine and muscimol (36, 99), have long been used to specifically target GABA_A mostly in mammalian cell types (104, 143). However, as discussed in Hamilton et al. (99) in 2013, methods to exclude possible effects on unintended targets have not been examined in marine fish. Many more GABA_A agonists and antagonists have been well characterized in mammalian models, and different GABA_A fish isofoms could show varied responses to pharmaceutical compounds based on subunit composition, as seen in mammals (see reviews in Refs. 132 and 208).

While measurements of HCO₃⁻ and Cl⁻ in fish plasma are available in the literature for fish exposed to high levels of CO₂ (8, 18, 39, 180), few studies have measured plasma HCO₃⁻ and PO₂ at levels relevant to ocean acidification projections (58, 215). Although species-specific differences are expected, the increase in plasma HCO₃⁻ relevant to CO₂ levels used in behavior and GABA-related studies (close to 1,000 μAtm CO₂) would be ~1–1.5 mM based on plasma HCO₃⁻ measurements in toadfish (58). In the brain (neuronal cells), the magnitude of this change could be even greater than expected.
since preferential regulation of pH\textsubscript{i} over pH\textsubscript{e} in vital organs, including the brain, has been documented in fish species at high levels of CO\textsubscript{2} (30,000–60,000 μAtm CO\textsubscript{2}) (8) and in muscle tissue of fish exposed to 1,900 μAtm CO\textsubscript{2} (58). At high levels of CO\textsubscript{2}, the regulation of pH\textsubscript{i} has been demonstrated to surpass the intrinsic intracellular buffering capacity and thus represents active regulation on the part of these tissues to carefully control pH\textsubscript{i} (8, 18). If this process is occurring in the brain at near future CO\textsubscript{2} levels, the time needed (2–4 days) to induce behavioral changes (157) could reflect the time course of mRNA expression and/or enzyme activity changes required to effectively compensate for a CO\textsubscript{2} acidosis (36).

Applying measured and predicted parameters from physiological studies on acid-base balance, GABA\textsubscript{A} receptor characterization, and electrochemical gradients allows for a theoretical examination of the idea that low-level hypercapnia could result in a reversal of current flow through GABA\textsubscript{A} receptors. The reversal potential for GABA\textsubscript{A} (E\textsubscript{GABA}) was calculated using estimates of extracellular and intracellular [Cl\textsuperscript{−}] and [HCO\textsubscript{3}\textsuperscript{−}] under control and 1,900 μAtm CO\textsubscript{2} conditions and compared with a commonly reported value of neuron membrane potential (−70 mV). Extracellular values for P CO\textsubscript{2}, pH\textsubscript{e}, and HCO\textsubscript{3} were based on measured and calculated values from previous studies (58) (Fig. 6). Intracellular [HCO\textsubscript{3} was then estimated using the Henderson-Hasselbach equation and appropriate constants (17) from measurements of pH\textsubscript{i} and assumed P CO\textsubscript{2} in white muscle of control toadfish and toadfish exposed to 1,900 μAtm for 24 h (58) (Fig. 6). Observations from white muscle pH\textsubscript{i} were considered to be representative of neuronal tissue and equilibrium of P CO\textsubscript{2} between tissues and blood was assumed for these estimates as in previous work (8, 20). The former is an assumption that would tend to err on the conservative side as brain tissue accumulates more intracellular HCO\textsubscript{3} than white muscle (8). Measured extracellular HCO\textsubscript{3} and estimated intracellular HCO\textsubscript{3} for the evaluation of E\textsubscript{GABA} under control and elevated P CO\textsubscript{2} conditions are reported in Fig. 6. To determine the reversal or equilibrium potential for the GABA\textsubscript{A} channel (E\textsubscript{GABA}), Cl\textsuperscript{−} gradients across the cell membrane as well as relative permeability of these ions through the GABA\textsubscript{A} channel were also considered. Extracellular Cl\textsuperscript{−} was assumed to be 150 mM, a value typical for marine teleosts (142), whereas intracellular Cl\textsuperscript{−} was assumed to be 22 mM, a value that is within the range reported from mammal, amphibian, and crayfish neurons (4, 9, 24, 47). For Cl\textsuperscript{−} concentrations under elevated P CO\textsubscript{2} conditions it is assumed that charge balance is maintained in both fluid compartments by adjustment of only HCO\textsubscript{3} and Cl\textsuperscript{−} concentrations (see Acid-Base Balance). Thus a 3 mM increase in [HCO\textsubscript{3}] is matched by a corresponding decrease in [Cl\textsuperscript{−}]. Since the gradients of these two ions are the only anion contributions to GABA\textsubscript{A}-mediated current under physiological conditions, E\textsubscript{GABA} could be calculated under control and elevated P CO\textsubscript{2} conditions according to the following equation (64):

\[
E_{\text{GABA}} = \frac{RT}{F} \ln \left( \frac{P_{\text{Cl}^{-}} [\text{Cl}^{-}]_{i} + P_{\text{HCO}_3^{-}} [\text{HCO}_3^{-}]_{i}}{P_{\text{Cl}^{-}} [\text{Cl}^{-}]_{o} + P_{\text{HCO}_3^{-}} [\text{HCO}_3^{-}]_{o}} \right)
\]

where P represents the relative permeability for HCO\textsubscript{3}/Cl\textsuperscript{−} through the GABA\textsubscript{A} channel, R is the ideal gas constant, T is the absolute temperature (25°C), and F is Faraday’s constant. The relative HCO\textsubscript{3}/Cl\textsuperscript{−} permeability (P) for GABA channels varies between 0.2 and 0.6 (64) and two representative levels of 0.3 and 0.4 were considered. The estimated impact of exposure to elevated CO\textsubscript{2} on E\textsubscript{GABA} is illustrated in Fig. 6. With the use of the commonly assumed resting neuronal membrane potential of −70 mV, the change in HCO\textsubscript{3} gradi- ents and resulting changes in Cl\textsuperscript{−} gradients could account for a reversal of the GABA\textsubscript{A}-mediated current from being hyperpolarizing and inhibitory under control conditions to depolarizing and excitatory under hypercapnic conditions (1,900 μAtm CO\textsubscript{2}). It is tempting to speculate that differences in sensitivity to CO\textsubscript{2} among sensory systems (see above) and among species could be related to differences in relative permeability for HCO\textsubscript{3}/Cl\textsuperscript{−} (P), which confers different E\textsubscript{GABA} values (Fig. 6). The GABA\textsubscript{A} receptor is typically composed of five subunits, and the β subunit is often most important for determining channel permeability properties (64). Subunit distribution has also been demonstrated to differ among brain regions (64, 110, 132), neurons (64), age (110), and development (110) in mammals, which might account for sensory system and/or species specificity in sensitivity. Ion permeability measurement and subunit characterization of GABA\textsubscript{A} isoforms in fish species with demonstrated behavioral impacts with CO\textsubscript{2} exposure would be informative.

In addition to basic changes in ion gradients, it is also interesting to consider the possibility that ocean acidification could lead to regulation of neuronal transporters and enzymes involved in HCO\textsubscript{3} and Cl\textsuperscript{−} transport. Developmental studies of rat hippocampal pyramidal cells demonstrate that a drastic upregulation of intraneuronal CA (CAVII) coincides with a depolarizing current induced by an increase in intracellular [HCO\textsubscript{3}] (196). Assuming fish neurons contain similar or comparable cellular machinery to other vertebrates, increases in intracellular HCO\textsubscript{3} could occur via Na\textsuperscript{+}-driven anion exchange (NDAE) or through increased hydration of intracellular CO\textsubscript{2} via CAVII (Fig. 6) (64). Both mechanisms could be occurring during CO\textsubscript{2} exposure, since blood P CO\textsubscript{2} and HCO\textsubscript{3} remain elevated (see Acid-Base Balance), thus providing substrate for both pathways. It is also possible that Cl\textsuperscript{−} current is mediating changes observed during hypercapnia; however, this current has usually been demonstrated to depolarize only in the early stages of development when intracellular Cl\textsuperscript{−} levels are high before K\textsuperscript{+}/Cl\textsuperscript{−} cotransporter (KCC2) membrane expression (196). It is important to note that reduced plasma Cl\textsuperscript{−} levels and/or increased cellular HCO\textsubscript{3} levels could lead to changes in intracellular Cl\textsuperscript{−} concentrations. Any alterations that lead to an upregulation of NKCC or a downregulation of KCC would likely promote depolarization (64). Both cation-chloride cotransporters (e.g., NCCs, NKCCs, and KCCs) and those that transport acid and base equivalents show variation in regulation, localization, and isoform in most mammalian models (15, 35, 48, 174), suggesting another avenue for further research in fish. Finally, as suggested in Hamilton et al. 2014 (99), regulation of GABA levels or GABA\textsubscript{A} receptor expression could also influence the dynamics of neurosensory disturbances in fish.

While many of the behavioral effects of CO\textsubscript{2} are well documented, exploration of the nature and time course of compensation during CO\textsubscript{2} exposure across species could greatly aid in quantifying fish sensitivity to ocean acidification. Furthermore, the link between these changes, GABA\textsubscript{A} receptor
function, and the effects of parental exposure should be explored in more detail.

**Growth, Development, and Survival**

Studies examining growth, mortality, and development report highly variable results, even among fish at similar life stages, CO₂ levels, or exposure durations (online supplemental Table 1). Several fish species have been found to be resistant to decreased growth, condition factor, and increased mortality from ~17,000 up to levels as high as 50,000 µAtm CO₂ (~8–25 times higher than ocean acidification predictions for year 2300) (72, 75, 102, 118, 126, 183) (see Ref. 117 for a detailed review of high CO₂ level exposures). Some of the variability noted across studies can arguably be related to differences in feeding rations as energy constraints may be offset by ad libitum feeding.
Furthermore, two recent studies have highlighted the importance of considering parental exposure (151) and life stage exposure (10) in designing experiments measuring growth, survival, and development.

**Growth.** Reported growth impacts, or lack thereof, do not show any particular patterns with respect to life history strategy or exposure duration. In studies focusing on embryonic or newly hatched individuals, reduced growth was noted in the estuarine inland silverside (780 μAtm CO₂) (10) and the cinnamon anemonefish (1,032 μAtm CO₂) (151), and a dose-dependent reduction in condition factor was demonstrated in juvenile Atlantic cod; however, this parameter was measured at higher CO₂ levels (1,000, 3,800, and 8,500 μAtm CO₂) (153). Several species including the Baltic cod (80), Atlantic herring (76), spiny damselfish (159), juvenile coral trout (163), walleye pollock juvenile yearlings (115), and cobia (14) have shown no change in growth parameters at CO₂ levels less than 2,000 μAtm. Adult fish measurements also show insensitivity, with no change in growth being reported for the three-spined stickleback at 900 μAtm CO₂ (121) and no change in body condition in the cinnamon anemonefish (152), even after 9 mo of exposure at ~1,030 μAtm CO₂ and an increase in reproductive output (see *Reproduction*). Finally, increased growth has been documented in the orange clownfish larvae (158) and the cinnamon anemonefish larvae (when parents were also exposed to 1,032 μAtm CO₂) (151). Subyearling juvenile walleye pollock also displayed an increase in growth; however, these fish showed no change in condition factor and no increase in weight-specific consumption rate, suggesting the increased growth was not due to an increase in food intake but rather changes in energy allocation (115). Feeding rate was also not found to be impacted by CO₂ (960 μAtm) in the cinnamon anemonefish (167). While some suggestions have been made to explain growth effects in particular species (see *Development*), it remains difficult to formulate common themes to explain the divergent patterns noted with respect to this endpoint. Miller and colleagues (151) demonstrated that parental CO₂ exposure can reduce or reverse deleterious effects on standard length and weight noted in acute transfer of offspring from control parents to high CO₂ conditions, a finding that could account for some variation seen in early life history growth parameters. Other studies suggest that growth should be examined in conjunction or in the context of other endpoints. Decreased RNA-to-DNA ratios (76), increased cortisol (183), decreased blood PO₂ (183), and increased serum glucose (183) serve as examples of noted impacts, while there was no significant change or an increase in growth.

**Development.** Studies assessing aspects of development also show variable results. Developmental delays have been noted in some species including cobia raised at higher CO₂ levels (3,500 μAtm) (14) and the Japanese ricefish at 1,200 μAtm (229). However, in the ricefish, this delay did not impact final environmental period; a result that was also evident in an increase in amino acid catabolism (see *Mitochondrial Function and Metabolic Pathways*) (229). In the cinnamon anemonefish, some compensation for developmental problems also appeared to occur if parents were exposed to ~1,030 μAtm CO₂ for 9 mo (151), since embryos from these parents showed no change in embryonic duration or length at hatch but did show reduced hatching yolk area (152). Orange clownfish also exhibited no change in embryonic duration and reduced yolk size; however, larval length was increased at the end of their pelagic stage (158).

Increases in developmental abnormalities have also been seen in certain species following CO₂ exposure. Increases in developmental abnormalities have also been observed in the two-spotted goby, despite no differences in embryonic duration (74). At least one study has shown increased lipid synthesis and tissue damage in 32 days posthatch Atlantic cod at 4,200 μAtm CO₂. Authors of this study suggested that the increased growth noted in this treatment was a direct result of the inability to appropriately divert energy away from growth to organ development; however, mortality rates were not directly measured (79). Overall, based on studies performed to date, development appears to be largely unaffected by CO₂ exposures, although a few notable exceptions exist. Future work detailing the ontogenetic timing of acid-base regulatory machinery in developing embryos, as noted in Tseng et al. (229) in 2013, could aid in generalizing or predicting developmental effects.

**Survival.** Despite the variation in species, exposure levels, and life stage, to our knowledge, only two studies to date on the inland silverside and cinnamon anemonefish have illustrated increased mortality rates in a fish species at ocean acidification relevant CO₂ levels (10, 151). Although the temperate silverside study demonstrated the importance of exposing species from the embryonic stage, studies on other species at this early life stage including the temperate Baltic cod (80), temperate Atlantic herring (76), and the tropical orange clownfish (158) have not demonstrated this same sensitivity. Studies on postembryonic and adult life stages are likely less sensitive, but it is still important to highlight that a wide range of species have been examined with no significant effects on mortality including the spiny damselfish (159), walleye pollock (115), cobia (14), cod (80, 153), and the three-spined stickleback (121). Baumann and colleagues (10) found that mortality was significantly increased when the inland silverside was exposed from the egg stage compared with a posthatch stage, demonstrating that using the embryonic life stage is likely necessary to assess CO₂ effects on survival.

**Reproduction**

It is likely that that ATP demanding compensation for acid-base balance disturbance (see *Acid-Base Balance*) comes at a price that could manifest as shifts in energy budgets and/or increases to baseline metabolism. Surprisingly, few studies to date have examined how ocean acidification may affect reproduction in fish, a process that is pivotal for population dynamics and energetically costly.

Whereas inhibition of sperm motility has been noted at high CO₂ tensions (~10,000 μAtm) (116, 243), no effect was found on cod sperm motility in the only study to date utilizing environmentally relevant CO₂ levels (78). Mating behavior was unaltered in the pipefish at pH 7.5, although it is important to note that the short-term exposure (4.5 h) of this study could have been insufficient to alter reproductive activity, since it typically takes ~4 days to observe behavior changes in other species measured (157) and complete acid-base compensation over this time period is unlikely (58).
Perhaps the most interesting and comprehensive study to date examining the effects of CO₂ exposure on fish reproduction at multiple life stages was performed by Miller and colleagues in 2013 (152), reporting an unexpected increase rather than decrease of reproductive output of adult anemone-fish. While this increase in reproductive output was expected to have an obvious consequence, condition factor of the spawning adults showed no difference across CO₂ treatments. Further examination of offspring also noted little evidence for an energetic consequence, since decreased egg area (584 μAtm CO₂) and decreased yolk size (1,032 μAtm CO₂) did not affect the length of newly hatched embryos (152) or impact routine metabolic rate, survival, and standard length in 31-day old juveniles from a parallel study (151). In fact, CO₂-exposed juveniles (1,032 μAtm CO₂) were significantly heavier than controls (151). Numerous plausible explanations that could be occurring in reproducing adults were suggested including hormesis, a stimulatory response that occurs at immediate exposure levels to a particular stressor, and is not necessarily favorable (26, 152). Other suggested explanations included increased energetic efficiency, delayed consequences over a lifespan, a decrease in routine metabolic rate, or changes to the reproductive neuroendocrine system (152).

Although studies have noted increased activity and/or boldness with CO₂ exposure that could arguably stimulate reproductive behavior, the authors of this study did not notice a difference between treatments (152). Study of reproductive output in other species is clearly needed to assess whether increased reproductive output is a ubiquitous response to ocean acidification and similar studies at higher exposure levels to a particular stressor, and is not necessarily favorable (26, 152). Other suggested explanations included increased energetic efficiency, delayed consequences over a lifespan, a decrease in routine metabolic rate, or changes to the reproductive neuroendocrine system (152).

Gamete production and associated reproductive behaviors are controlled by the hypothalamo-pituitary-gonadal (HPG) axis (169, 242), and ocean acidification may either be stimulating a portion of this axis, as suggested in Miller et al. (152) in 2013, or causing a lack of inhibition where one should be present. Interestingly, the same GABA_A receptors that have been associated with disruptions in fish olfaction, behavioral lateralization, anxiety, and learning with ocean acidification (166) (see Neurosensory and Behavior) may also influence the HPG axis in fish. GABA and/or its agonists have been found to stimulate the release of luteinizing hormone in at least three fish species (123, 125, 127, 139) and has also been demonstrated to inhibit dopamine release (228), both of which could stimulate reproductive pathways. However, it is important to note that these effects may be specific to reproductive life stage, the species, and sex of the fish and dependent on levels of sex steroids (127, 228, 242). Considering the robust link established between GABA_A receptors and other behaviors (see Neurosensory and Behavior), examining how CO₂ affects GABA levels and GABA_A receptors in the HPG axis could shed more light on the unexpected increase in reproductive output observed during exposure to elevated CO₂.

**Metabolic Rate and Swim Performance**

The difference between standard metabolic rate (SMR) and maximum metabolic rate (MMR) measured as oxygen consumption is termed aerobic scope and is hypothesized to relate to whole animal fitness and performance (see Ref. 41 for a recent review). Two types of stresses, loading stress and limiting stress, may limit aerobic scope and thus reduce animal fitness (21). Loading stress adds to the SMR while limiting stress acts to reduce the MMR (21). Several studies have assessed the potential impact of elevated PCO₂ on aerobic scope and metabolic rates with the general assumption that the cost of coping with elevated PCO₂ (acid-base balance, osmoregulatory, and cardiorespiratory adjustments) would increase SMR (loading stress) and thus reduce aerobic scope and thereby fitness. Two studies to date, one on tropical reef fish species and one on four Antarctic fish, all exposed to ~1,000 μAtm, have confirmed this expectation and demonstrated apparent increase in SMR (57, 155), although the latter study also reports data from species showing no apparent CO₂-induced elevation in SMR. In contrast, six studies report no apparent change in tropical, temperate, and Antarctic species (43, 46, 147, 151, 215), and one even reports an apparent decrease in SMR in a tropical reef fish (202) following exposure to PCO₂ levels ranging from 860 to 10,000 μAtm when elevated CO₂ was considered without temperature manipulation.

Elevated ambient PCO₂ has been hypothesized to potentially reduce oxygen uptake and delivery (limiting stress) due to acidification of blood and tissue respiratory pigments (190). While most species studied to date are capable of regulating intra- as well as extracellular pH (see Acid-Base Balance), it should be noted that our knowledge of acid-base balance in deep sea and cold environment species is modest and that these species may in fact be limited in their pH regulatory ability (187). The hypothesis of a CO₂-induced reduction in MMR has been tested repeatedly with mixed results. A single study on tropical reef species exposed to ~1,000 μAtm confirms the hypothesis of reduced MMR (155), whereas one report on the temperate Atlantic cod showed no impact at levels up to 6,000 μAtm. Surprisingly, two studies report increased rather than decreased MMR in tropical reef species exposed to ~900 μAtm (43, 202). The unexpected increase of MMR during CO₂ exposure has been suggested to be the result of a stressor or mild acidosis, which may induce catecholamine release and stimulation of RBC Na⁺/H⁺ exchange to elevate intracellular pH thus facilitating hemoglobin-O₂ binding at the gill and also possibly facilitate oxygen delivery to CO₂-producing tissues via root effect hemoglobin (43, 200, 201).

Considering the variable reports of CO₂ impact on SMR and MMR, it is not surprising that studies report reduced aerobic scope (155), unaltered aerobic scope (147), and even increased aerobic scope (43, 202) following exposure to low levels of CO₂.

It is clear from studies performed at the same facilities, applying near-identical techniques (43, 155, 202) that substantial difference exists among species with respect to how SMR, MMR, and thus aerobic scope are impacted by elevated PCO₂. Furthermore, it seems clear that interactions between PCO₂ and ambient temperature may affect these PCO₂ responses (57, 155). However, there can be little doubt that technical and experimental differences contribute to the variable responses.
reported in the literature. Measurements of aerobic scope are inherently difficult. Standard metabolic rate (the metabolic rate associated with baseline activity in fasting animals) cannot be directly measured and rather must be derived from either swim tunnel respirometry (21, 22), where oxygen consumption measurements performed at different activity levels allows for extrapolation to baseline activity, or by assessment of frequency distribution of routine metabolic rates, which includes a level of spontaneous activity, as discussed recently (41). Unfortunately, several of the studies cited above are basing assessments of CO2 impacts on aerobic scope on routine, rather than standard, metabolic rates, which are subject to substantial variability under any circumstance due to the inclusion of spontaneous activity. The clear and well-documented impacts of CO2 on fish behaviors such as increased boldness and activity levels (see Neurosensory and Behavior) could amplify the metabolic cost of spontaneous activity in CO2, but not control exposures, leading to further complication in assessing routine metabolic rate. Furthermore, it should be noted that routine metabolic rates, as assessed by respirometry, can be highly variable even at what appears to be constant activity levels due to differences in anticipatory and anxiety related responses to ambient conditions (light, presence of experimenters, etc.) (212, 213).

Similar problems exists with determination of MMR as recently demonstrated by side-by-side comparisons of distinct methods [chase to exhaustion, chase to exhaustion followed by air exposure, and finally swim tunnel respirometry (197)].

Overall, it appears that swim tunnel respirometry, which has been applied in only one study to date showing no effects of CO2 on cod swimming performance (147), is the more consistent method for determining aerobic scope as it provides the standard and maximum metabolic rates (197). However, swim tunnel respirometry is not suited for sedentary species that will not swim continuously when placed in a laminar water flow. Furthermore, swim tunnel respirometers are costly and may therefore not be practical for high throughput experiments. For studies applying standard respirometers, the authors of the present article agree with the recommendations of using intermittent flow respirometry (212, 213) and avoiding the use of routine oxygen consumption (41) for calculations of aerobic scope.

Three studies assessing maximal swim performance (U_{max}) found no effects of up to 6,000, 2,100, and 1,030 μAtm on the temperate Atlantic cod, subtropical cobia, and on a tropical clownfish, respectively (14, 147, 158). Furthermore, cobia larvae display no effect of elevated Pco2 on spontaneous and olfactory-stimulated swimming activity (14). Similarly, a study examining spontaneous swimming activity on Atlantic cod found no appreciable effects of CO2 levels as high as 4,200 μAtm, although subtle changes in the stop-and-go swim pattern characteristic for larvae of this species were observed and may have implications for prey encounters and energy conservation (140).

Mitochondrial Function and Metabolic Pathways

Since alterations of enzyme activity and metabolic pathways involved in mitochondrial function can be reflective of aerobic scope, understanding how environmental factors alter mitochondrial metabolism can be important in predicting aerobic performance and overall fitness at future CO2 levels (185, 186, 189, 217). This section will specifically focus on changes noted in response to CO2 exposures, but it should be recognized that impacts of temperature and CO2 in combination are more relevant for predicting effects of climate change scenarios [see concept and discussion of oxygen and capacity limited thermal tolerance (O.C.L.T.T.) in Portner reviews (185, 186, 189)].

Changes in mitochondrial enzymes and metabolic pathways in fish following prolonged CO2 exposure have been examined in multiple tissues in an adult Antarctic fish (Nototthenia rossii) (215–217), a temperate fish (Sparus aurata) (150), and also in embryonic and larval Japanese ricefish (Oryzias latipes) (229). In N. rossii, liver mitochondrial state III respiration (215, 216), cytochrome c oxidase (COX) activity (215), complex I and complex II respiration (216), and the respiratory control ratio (RCR) (215) were all found to decrease with CO2 exposure (~2,000 μAtm CO2), indicating that mitochondrial pathways were altered and mitochondrial metabolic capacity was decreased (215–217). Despite these changes, organismal level parameters including routine metabolic rate (RMR), hepatosomatic index (HSI), and condition factor (215) were not significantly affected by CO2 exposure. Interestingly, some degree of compensatory response during metabolic shifts has been suggested in certain tissues, including the liver (216), red muscle (217), and heart (217). Increased P/O ratios (ADP produced/oxygen consumed) in hepatic complex I during CO2 exposure indicates such a compensatory response (216). In a parallel study examining COX and citrate synthase (CS) activity in heart, red muscle, white muscle, and liver, COX activity, CS activity, and COX/CS ratios were found to be significantly higher in the red muscle, and CS activity was significantly elevated in the heart (217), whereas COX activity in the liver was decreased (217). Together, these findings were suggestive of a compensatory response or a tradeoff, whereby metabolic adjustments in the liver may facilitate the increase in metabolic capacity that is necessary in the red muscle and the heart during hypercapnic stress (217). Despite this evidence, this compensation response may only be partial and unsustainable over longer time scales if these heightened energetic demands exhaust energy reserves in the liver (216, 217).

It has been hypothesized that the underlying mechanism causing metabolic shifts and changes to metabolic capacity in the liver could be a result of elevated HCO3− and Pco2 levels associated with acid-base compensation that could inhibit a portion of the tricarboxylic acid (TCA) cycle (216, 217). Although protons in the mitochondria could react with other processes, increased pyruvate kinase (PK), increased lactate dehydrogenase (L-LDH), decreased CS, increased malate dehydrogenase (MDH), and increased 3-hydroxylacetyl CoA dehydrogenase (HOAD) in various tissues including the red muscle, white muscle, and heart also suggested shifts in metabolic pathways (150). These changes, in combination with
increased plasma lactate, indicated some degree of change from aerobic to anaerobic metabolism in this species (150).

Downregulation of metabolically relevant genes (G6PDH-glucose-6, CS, cytochrome c) in embryonic Japanese ricefish also indicate a shift in metabolic pathways, although this trend was less robust in newly hatched larvae (229). Expression levels of genes implicated in amino acid catabolism showed a reversal from general downregulation to upregulation from embryonic to newly hatched life stages, suggesting that these fish start consuming amino acids to meet energetic needs during hypercapnia exposure after hatch (229).

Evidence of metabolic shifts and compensatory responses, along with species differences in metabolism clearly show that this area of research is crucial and valuable in understanding underlying physiological mechanisms that dictate an important aspect of energetic allocations in fish. Future studies examining the effects of ocean acidification on mitochondrial pathways should include more tropical, subtropical, and temperate species and continue to investigate links to organism level metabolic consequences.

Cardiorespiratory Responses to Elevated CO₂

Water-breathing fish tend to adjust ventilation in response to environmental O₂ levels and primarily use metabolic adjustments to cope with acid-base disturbances (see Acid-Base Balance). Nevertheless, whereas metabolic adjustments during a CO₂-induced respiratory acidosis are likely the dominant acid-base compensation mechanism, many studies dating back to the 1970s have demonstrated a hyperventilatory response to hypercapnia (see Refs. 85 and 86 for relevant reviews). This hyperventilatory response is often characterized by an increase in ventilatory amplitude and/or an increase in ventilation frequency, although these responses can vary widely across species and minimum threshold CO₂ levels (85, 86). Since ventilatory adjustments for water breathers would generally be considered more energetically costly than metabolic modification due to the viscosity of the aquatic medium, there has been some speculation to explain this response. It has been suggested by Gilmour in 2001 (86) that hyperventilation could decrease the drop in pH experienced during a CO₂ acidosis (85, 86, 177), serve as an “early warning system” for hypoxia (85, 86), or aid in restoring the “O₂ debt” incurred after intense exercise (238). In addition to hyperventilation, bradycardia is also a common cardiovascular response to hypercapnia but is not present in all species (85, 177). The proposed function of bradycardia is still debated, especially since its interpretation can depend on other cardiovascular parameters such as stroke volume, changes in vasculature, and blood pressure (see Ref. 177 for a detailed review).

Much of the research examining hypercapnic hyperventilation has been performed utilizing relatively high levels of CO₂ (10,000–50,000 μAtm CO₂). However, it has recently been demonstrated that levels as low as ~1,300 μAtm CO₂ induce hyperventilation in zebrafish (freshwater) (230), suggesting some fish species may perform respiratory as well as metabolic adjustments at environmentally relevant CO₂ levels. As ventilatory modifications are typically more energetically costly, using this strategy could have implications for baseline metabolic costs.

Since fish likely detect CO₂ in the environment to make cardiorespiratory adjustments, considerable research has focused on branchial chemoreceptors known as neuroepithelial cells (NECs). Initial studies of NECs focused on their ability to sense oxygen (55, 120, 191); however, recent research on zebrafish has shown that ~30% of NECs that respond to changes in O₂ also respond to CO₂ (10,000 μAtm), suggesting some of these cells are capable of sensing changes in external CO₂ (191). Similar to NEC sensing of hypoxia, the proposed mechanism for sensing hypercapnia involves an inhibition of K⁺ channels that causes accumulation of intracellular K⁺, depolarization, Ca²⁺ cellular entry, and finally the release of neurotransmitters (1, 177, 191). At present, information on this topic is restricted to freshwater species pointing to a clear need for additional work on marine fish.

Assuming other fish species exhibit respiratory adjustments at near-future CO₂ tensions, it is important to consider cardiorespiratory acclimation under chronic CO₂ exposure, especially since zebrafish have been demonstrated to show some degree of plasticity according to life stage (231) and exposure duration (230). Hypercapnia preexposure in embryos (7 days) did not affect acute response to hypercapnia in adult zebrafish (231); however, prolonged exposure to hypercapnia in adults (28 days) blunted subsequent acute responses to CO₂ (230). Certain NEC types have also been demonstrated to increase in size and density (120) during chronic hypoxia exposure (60 days). Since some NECs respond to both hypoxia and hypercapnia, this response could also be noted during exposure to elevated CO₂. However, chronic hypercapnia was not found to change the size or density of examined NECs in zebrafish, although the authors of this study acknowledge certain relevant cell types may have been overlooked (230).

Given the predicted increases in CO₂ over the next two centuries and the likelihood that fish living in coastal areas may see acidification and hypoxia exacerbated by excessive nutrient runoff (25, 65), studies of cardiorespiratory sensing and responses to sustained hypercapnia and hypoxia are necessary. Research performed to date on the cardiorespiratory responses to different permutations of these combined stressors has shown mixed results, including no effect (231), “blunting” (230), and additive effects (16). These results, combined with the high variability seen in cardiorespiratory parameters in response to both hypercapnia and hypoxia, indicate more research examining interacting effects of these stressors across species and at lower CO₂ exposure levels is warranted.

Osmoregulation

Marine teleosts maintain plasma osmolality at or slightly higher than 300 mosM and as a consequence experience a continuous diffusive water loss to their surrounding saline environment. This water loss is compensated for by seawater ingestion and fluid absorption across the intestinal epithelium. The fluid absorption is driven by the absorption of NaCl. Other processes that reduce the osmotic pressure of the intestinal fluids also promote water absorption and include CaCO₃ precipitation and to a lesser extent titration of luminal HCO₃⁻ by secretion of acid by the intestinal epithelium (see Refs. 90, 92, and 96 for recent reviews). The excess NaCl gained by the process of intestinal fluid absorption is eliminated by the gills while the excess MgSO₄ is excreted with urine (142).
Up to 70% of Cl− uptake by the intestinal epithelium is accounted for by anion exchange (apical “A6” Fig. 5) and a significant portion of the HCO₃⁻ secreted into the intestinal lumen is derived from the blood plasma (89, 93). As fish perform metabolic compensation for elevated CO₂ (see Acid-Base Balance), increased plasma HCO₃⁻ leads to increased HCO₃⁻ transport from the blood into the intestinal epithelial cell via basolateral NBC1 (221) (Fig. 5). This increased HCO₃⁻ uptake across the basolateral membrane provides additional HCO₃⁻ for apical HCO₃⁻ excretion to the lumen and thus Cl− uptake by the intestinal epithelium as evident from elevated [HCO₃⁻] and reduced [Cl−] in rectal fluids collected from toadfish exposed to 1,900 μAtm (111). The increase in intestinal Cl− uptake likely is due in part to increased anion exchange; however, the depletion of rectal fluid Cl− is greater than the corresponding increase in HCO₃⁻. This discrepancy occurs despite the nHCO₃⁻/Cl− stoichiometry of the SLC26a6 anion exchanger and may suggest that additional Cl− uptake pathways are stimulated by elevated CO₂. A candidate pathway for additional Cl− uptake is the apical NKCC2 isofrom (Fig. 5), which is stimulated by elevated HCO₃⁻ via the soluble adenyl cyclase (sAC) (227). In agreement with this suggestion is a trend toward reduced rectal fluid [Na⁺] in toadfish exposed to 1,900 μAtm (111). Regardless of the pathway for Cl− (and Na⁺) uptake, the increased ion gain must be compensated for by the gill through extrusion of these ions.

Among several queues, reduced [Cl−] in the intestinal lumen stimulates seawater ingestion rates (6, 113), and the hypothesis that reduced luminal Cl− during exposure to elevated CO₂ would lead to stimulated anion exchange was tested on toadfish exposed to 1,900 μAtm, which showed a trend for increased drinking rates (111). Seawater ingestion by marine fish is variable over time and a highly dynamic process under control by a wide range of factors (92). Additional studies on the potential impact of elevated CO₂ on drinking rates are needed since stimulated drinking could elevate baseline metabolic costs.

Regardless of potential effects of CO₂ on drinking rates, branchial NaCl extrusion is driven by NKA with Na⁺ moving via the paracellular pathway and Cl− entering the cell via basolateral and leaving across the apical membrane into seawater via apical cystic fibrosis transmembrane conductance regulator (CFTR) channels (Fig. 5). Thus, considering that intestinal Cl− (and possibly Na⁺) uptake is increased during CO₂ exposure, it is not surprising that NKA mRNA expression, protein content, and maximum enzymatic activity in eelpout gill tissue increased during exposure to 10,000 μAtm (46), that NKA protein expression and enzymatic activity increased in gill issue of Atlantic cod exposed to 6,000 μAtm (147), and finally that NKA activity increased in gill tissue of Olive flounder exposed to −100,000 μAtm (101). Despite using CO₂ levels unrealistic for ocean acidification predictions, these reports of increases in branchial NKA abundance and activity could reflect an increased intestinal Cl− and possibly Na⁺ uptake and thus a need for additional ion excretion across the gill epithelium. In addition, elevated branchial NKA activity could reflect an increased demand for Na⁺/H⁺ and Cl−/HCO₃⁻ exchange as well as Na⁺/HCO₃⁻ cotransport processes in the gill tissue as discussed above (see Acid-Base Balance). However, it should be noted that at least two studies on toadfish exposed to 1,900 μAtm (58) and Atlantic salmon smolts exposed to 20,000 μAtm (205), respectively, report reduced rather than increased NKA activity. It is unclear whether this diversity of responses reflects species differences, PCO₂ levels, exposure duration, or conflicting compensatory responses to osmoregulatory and acid-base balance challenges (see Acid-Base Balance).

**Perspectives and Significance**

It is clear that fish are impacted by low-level CO₂ exposure across a broad range of effect categories (Fig. 2). Though most of these impacts do not result in direct mortality, sublethal effects can affect fitness of individuals and extend across populations and communities. From the present review, it appears that the majority of studies to date reporting significant CO₂ effects at environmentally relevant CO₂ levels focus on neurosensory and behavioral impacts in tropical species. Around 70% of reported CO₂ effects from all categories are from studies performed above 22°C (10°C range), whereas studies on species in cooler temperatures ranging from −3 to 22°C (25°C range) only make up ~30% of reported effects (Fig. 7). While biodiversity largely increases with decreasing latitude (see review in Ref. 112), this basic analysis does suggest that temperate and polar species and associated environments are understudied. It is also clear that more research is needed at longer study durations and on earlier life stages,
which are generally perceived to be more sensitive to environmental perturbation. Embryonic and larval life stages comprise ~35% of all reported significant effects and only ~30% of reported significant effects were performed using exposure durations greater than 10 days (Fig. 7).

Currently, many explanations for noted CO₂ effects on fish (neurosensoric, behavioral, otolith growth, and metabolic rate) appear to hinge upon changes expected to occur during acid-base balance compensation. However, few studies (58, 215) have characterized the nature and time course of this disturbance in extracellular and intracellular compartments at ocean acidification relevant CO₂ levels. Furthermore, little is known about the downstream effects and variation in dynamic regulation in response to the increase in PCO₂ and HCO₃⁻ that occurs in the body to defend pH₃ and pH₅ at ocean acidification relevant CO₂ levels. Some general trends in cellular transport pathways involved in acid-base balance following CO₂ exposure are emerging, but many transporters and enzymes show variability in response and others have been understudied at low CO₂ levels. Differential cellular “strategies” may be utilized depending on species, exposure time, exposure level, and temperature. The variation in minimum CO₂ thresholds needed to alter otolith formation and the absence of an effect in certain marine fish provide an excellent example of a trait that would benefit from further knowledge of acid-base status and characterization. Although it has been established that otolith formation in teleosts likely relies on blood HCO₃⁻ and avoid excessive CO₂ as substrate and that HCO₃⁻ transport is carrier mediated, very little is known about the specific transporters involved in translocation of base and acid equivalents across the saccular epithelium. Isolating this tissue for molecular and in vitro physiological study and establishing concrete links between increased otolith growth and auditory ability could enhance this research area.

The majority of current research suggests CO₂ levels expected before 2100 will induce drastic and disorienting neurosensoric disruptions in fish if adaptation does not keep pace with the rate of environmental change. The pervasiveness of these issues across sensory systems and endpoints like learning and lateralization, provide strong evidence for broader neurological malfunction. Emerging research implicating a current reversal in the ubiquitous GABA_A receptor linked with a resulting divergence in behavior aligns well with this assessment and theoretical estimations of E_GABA presented above. This reversal from hyperpolarizing to depolarizing current is presumably linked to altered Cl⁻ and HCO₃⁻ gradients and serves as another instance where study of acid-base compensation response could offer needed insight. The wide body of literature on GABA_A and GABA in both whole animal and isolated systems in other species (64) could be a good starting point for designing future physiological studies on the neurophysiology of fish exposed to hypercapnia. Such studies ought to include measurements on extracellular as well as intracellular HCO₃⁻ and Cl⁻ concentrations and preferably neuronal membrane potentials. The potential for dynamic regulation in neurons along with recent evidence of parental CO₂ exposures dampening neurological effects in CO₂ offspring should also be examined more thoroughly. Similarly, parental exposure also appears to be an important component in studies examining growth, survival, and development, a topic area which despite a significant number of studies (online supplemental Table 1), displays few clear effect patterns. In contrast, studies examining the effects of CO₂ on fish reproduction are rare. One study has demonstrated an unexpected increase in reproductive output with no apparent associated cost. Authors of this study provide a number of interesting suggestions to account for this increase and previous studies on the involvement of GABA and GABA_A receptors in fish reproduction provide a logical avenue for future research. Examining reproduction during CO₂ exposure in other species under detailed feeding rations representing a variety of conditions including restricted food availability may also help clarify if tradeoffs are occurring under CO₂ conditions that are undetectable at rations that maintain or promote growth.

Reports of standard and maximum O₂ consumption, aerobic scope, and swim performance following CO₂ exposure vary widely across studies and species. It is clear that some of this variation is due to interstudy variation in methodologies, especially since assessing values needed to calculate aerobic scope are inherently difficult in species not amenable to swim tunnel respirometry. Furthermore, whole animal measurements of oxygen consumption (standard, routine, and maximal) are subject to considerable variation resulting in variable aerobic scope measurements regardless of the methods employed. These variations, which are mainly due to anxiety and/or anticipatory responses, combined with a low presumed metabolic cost of acid-base/ion exchange (<10% of basal metabolic rate) makes it challenging to firmly establish CO₂ impacts on metabolic rates of intact animals. Assessment of metabolic cost of CO₂ exposure using isolated tissue preparations and examining changes at the cellular and biochemical level as discussed below and in Mitochondrial Function and Metabolic Pathways might provide fruitful alternatives.

Low-level hypercapnia appears to cause changes in mitochondrial and metabolic pathways within specific body tissues and across species; however, some degree of compensation is likely at the organismal level. The effects of elevated HCO₃⁻ and PCO₂ are hypothesized to have a direct impact on specific functional components of the mitochondria (see Mitochondrial Function and Metabolic Pathways). The mechanistic physiology employed in this area of research is impressive and should continue, especially since energetic costs and effects on metabolism may be visible at this level of organization that may not be overtly evident at the organismal level for reasons discussed above. Nonetheless, even small increases to baseline costs or energetic reallocations can manifest at the population level.

Although hyperventilatory responses have been noted in certain fish species in response to hypercapnia, research on this response at ocean acidification-relevant CO₂ levels is currently restricted to freshwater species. Although more details regarding the CO₂-sensing mechanisms are emerging and many studies have examined cardiorespiratory endpoints during combined hypercapnia and hypoxia stressors, species variability is high and the long-term effects of low-level hypercapnia remain unknown in marine fish.

Although existing evidence does not support the idea that the marine fish intestine aids in dynamically regulating acid-base balance, it is clear that there are impacts on cellular transport mechanisms during hypercapnia. Since marine fish are dependent on drinking seawater and this process involves the exchange of acid and base equivalents both for uptake at the
intestine and excretion at the gill, it is not unreasonable to suspect that hypercapnia could interact/interfere with osmoregulatory homeostasis and increase baseline metabolic costs of either process. These interactions should be revisited at realistic CO2 levels in light of current predictions.

Finally, focus is required to assess the potential for fish populations to adapt (157) to future CO2 levels. Large variation among individuals in test groups at intermediate CO2 levels close to 700 μAtm has now been confirmed in six species to date (68, 157, 161, 163) and provides a target for natural selection and thus adaptation. On this background, adaptation undoubtedly will occur (and possible has occurred already) and needs to be examined. Perhaps even more importantly, the tradeoffs, including metabolic costs, of such adaptation (as well as acclimation) must be examined to ascertain if increased tolerance to elevated CO2 is associated with a reduced ability to cope with other environmental stressors and reduced overall fitness.

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