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Digestion under Duress: Nutrient Acquisition and Metabolism during Hypoxia in the Pacific Hagfish

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ABSTRACT

Hagfish feed by immersing themselves in the body cavities of decaying animals. This ensures a rich nutrient source for absorption via the gills, skin, and gut, but it may also subject hagfish to reduced levels of dissolved oxygen and elevated levels of the products of biological degradation. This study investigated the impacts of hypoxia and ammonia on the assimilation and metabolism of selected nutrients (glycine, L-alanine, and glucose) in Pacific hagfish (*Eptatretus stoutii*). Throughout exposure to hypoxia, plasma glucose levels increased. This was not accompanied by an increase in gut glucose transport, which suggests mobilization of glucose from body glycogen stores. Hypoxia preexposure enhanced glycine absorption across the gut and the gill, although L-alanine uptake was unchanged in these tissues. A 24-h period of exposure to hypoxia in hagfish concurrently exposed to waterborne radio-labeled glycine led to a large (5.7-fold) increase in brain glycine accumulation. Preexposure to high levels of waterborne ammonia (10 mM) for 24 h had no impact on gut or skin glycine uptake. These results indicate that hagfish are adapted to maintain nutrient assimilation despite environmental stressors and that tissue-specific absorption of key nutrients such as glycine can even be enhanced in order to sustain critical functions during hypoxia.

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Introduction

Hagfish are among the most unusual of all vertebrate animals. They are the only known osmoconformers among this group (Currie and Edwards 2010), they display a low-pressure circulatory system complete with accessory hearts (Forster 1998), and they are capable of exuding copious quantities of slime as a defense mechanism (Fudge et al. 2005). The distinctive characteristics of hagfish also extend to feeding physiology. Some indications suggest that they are predators of invertebrates, but they have also been observed scavenging on decaying carcasses that reach their seafloor habitat (Martini 1998). For large food items, they may enter the body cavity and eat their way out (Martini 1998). This exposes their skin and gill epithelia to enriched levels of dissolved organic nutrients; recent evidence shows that hagfish are unique among vertebrates in that they are capable of absorbing amino acids across these surfaces (Glover et al. 2011*a*). However, such a feeding environment is also likely to expose the hagfish to reduced dissolved oxygen levels. Hypoxia will be further exacerbated by extensive slime extrusion, which is used as an anticompetitive mechanism while feeding (Tamburri and Barry 1999). It is also probable that this in situ feeding environment will be highly enriched with products of biological decay, such as ammonia. As hagfish are opportunistic feeders and competition for temporally and spatially limited resources is high, the capacity to maintain nutrient acquisition in light of significant co-occurring stressors such as elevated ammonia and hypoxia is critical.

While little is known regarding ammonia tolerance in hagfish, these animals are known to be tolerant to hypoxia. Most hagfish burrow, and their capacity to withstand reduced environmental oxygen likely reflects this habitat (Forster 1998). Furthermore, the natural waters inhabited by hagfish are increasingly subjected to periods of anoxia (Chan et al. 2008). Physiologically, adaptations such as an extremely low metabolic rate (Forster 1990) and a feeding musculature (Baldwin et al. 1991) and cardiovascular system (Cox et al. 2010*a*) with enhanced capacities for prolonged anaerobic metabolism are thought to facilitate tolerance to reduced environmental oxygen. As feeding in hagfish likely coincides with exposure to hypoxia and high environmental ammonia, we hypothesized that a further adaptation would be the maintenance of nutrient absorption under these conditions.

Exposure to environments that push physiological and biochemical systems to their limits often coincides with modification of energy-dependent processes such as nutrient acquisition. In aquatic animals, feeding may cease at very low oxygen tensions (Bernatis et al. 2007) and hypoxia may slow digestive processing (Jordan and Steffensen 2007; McGaw 2008). Hyp-

oxia has been shown to reduce assimilation efficiency in fish (Zhou et al. 2001), while in mammals hypoxia-induced inhibition of the absorption of nutrients such as amino acids (Nelson et al. 2003) and glucose (Kles and Tappenden 2002; Schoots et al. 2006) has been described.

This study tested the hypothesis that hagfish have developed mechanisms that allow them to maintain nutrient assimilation despite exposure to conditions such as elevated ammonia levels and reduced oxygen during feeding. A small number of nutrients (glucose, glycine, and L-alanine) were selected for investigation. The amino acids were chosen largely on the basis of earlier studies characterizing their uptake across hagfish epithelia (Glover et al. 2011*a*, 2011*b*). In addition, hagfish muscle is known to accumulate high levels of glycine and L-alanine (Robertson et al. 1991), and these amino acids are both known to offer cellular protection against hypoxia-induced damage (e.g., Brecht and de Groot 1994). We hypothesized that these amino acids may therefore be particularly important during hypoxic exposure in hagfish and that this may be reflected in their nutrient uptake profiles. Glucose was examined owing to the known reliance of the hagfish cardiovascular system on carbohydrate metabolism (Forster 1998), meaning that any environmental glucose would act as a potential source to sustain the animal through hypoxia.

Gastrointestinal absorption of glucose and the uptake of the amino acids glycine and L-alanine across the gill, skin, and gut epithelium were investigated using a number of in vitro techniques in hagfish subjected to prior exposure to hypoxia and/or waterborne ammonia. On the basis of these experiments, the possible role of glycine in hypoxia tolerance was further investigated using in vivo waterborne exposure to radio-labeled amino acid.

Material and Methods

Animal Collection and Euthanasia

Pacific hagfish (*Eptatretus stoutii*) were captured from Barkley Sound, Vancouver Island, Canada, using baited traps, and then were transferred to outdoor holding facilities (tarpaulin-covered 5,000-L flow-through tanks, 12°C, natural summer lighting) at Bamfield Marine Science Centre (BMSC). Fish were held unfed for at least 7 d before experimentation. For collection of tissue, hagfish were euthanized by anesthetic overdose (3-aminobenzoic acid ethylester [MS-222]; Sigma, St. Louis, MO; 2 g L^{-1}). All manipulations were approved by the BMSC animal ethics committee (approval RS-10-10).

Hypoxia Exposure

For studies focusing on glucose metabolism, hagfish were placed in individual exposure chambers (800-mL airtight plastic containers) containing hypoxic water (bubbled with N_2 until Po_2 was in the range of 18–28 mmHg). As hagfish consumed the available oxygen, water Po_2 continued to decline, and it reached an undetectable level by the conclusion of the exposure. Every 6 h fish were removed and sacrificed, and blood was sampled from the main heart for glucose concentration (see below) and oxygen partial pressure (Radiometer-Copenhagen electrode; Copenhagen). In animals sampled at 24 h, gut glucose uptake was assessed using the double-perfused gut sleeve technique described below. Control animals were exposed to identical conditions, except with water flowing through their holding chambers to ensure normoxia.

To test the impact of hypoxia on amino acid transport, an identical protocol was followed except animals were sacrificed only at 24 h. Again an identically treated normoxic control group was included. Gut, skin, and gill tissues were removed and amino acid uptake in these tissues was measured via the techniques described below. Amino acid (glycine and L-alanine) absorption was determined at levels of 1 mM for gut tissue and 50 μM for skin and gill tissue. These levels represent concentrations within the linear portion of concentration-dependent kinetic curves for each tissue (Glover et al. 2011*a*, 2011*b*).

Ammonia Exposure

Preliminary experiments showed that Pacific hagfish were highly resistant to waterborne ammonia, capable of withstanding exposure levels of 10 mM ammonia for at least 96 h. A "worst-case scenario" of exposure was tested by subjecting adult hagfish ($n = 6$) to 10 mM ammonia (as ammonium chloride; at pH 8.0 and a temperature of 12°C). This represented a unionized ammonia (NH_3) level of 167 μM (Cameron and Heisler 1983). A control group was held under identical conditions except for the absence of added ammonia. Ammonia concentrations in decaying seafloor carcasses are unknown, and there is little information regarding ammonia levels in marine benthic environments; however, levels in estuaries may occasionally peak in excess of 10 mM (Eddy 2005). Hagfish were exposed for 24 h before removal of skin and gut tissue and assessment of glycine uptake (see below). Uptake was assessed at two concentrations of glycine in each tissue, 0.01 and 0.50 mM for skin and 0.50 and 10 mM for gut, on the basis of kinetic characteristics for glycine previously determined in these tissues (Glover et al. 2011*a*, 2011*b*). The lower level tested was in the linear portion of the uptake curve, while the higher value was in the saturating part of the curve. Uptake was determined in the absence of ammonia in the assay medium.

In Vitro Double-Perfused Gut Sleeves for Glucose Uptake Determination

Glucose absorption in hagfish gut was investigated using a novel in vitro gut perfusion technique (Fig. 1). Following an incision that exposed the viscera, a flared PE50 cannula was inserted in the major ventral gut blood vessel approximately 5–10 cm distal to the major hepatic vessel and secured in place with surgical silk. A second cannula was similarly inserted in the dorsal gut blood vessel connecting the enteric circulation with the liver. This created an artificial circulatory system whereby serosal ("blood") solutions could perfuse via the afferent (first) cannula, pass through the minor vessels of the intestine connecting

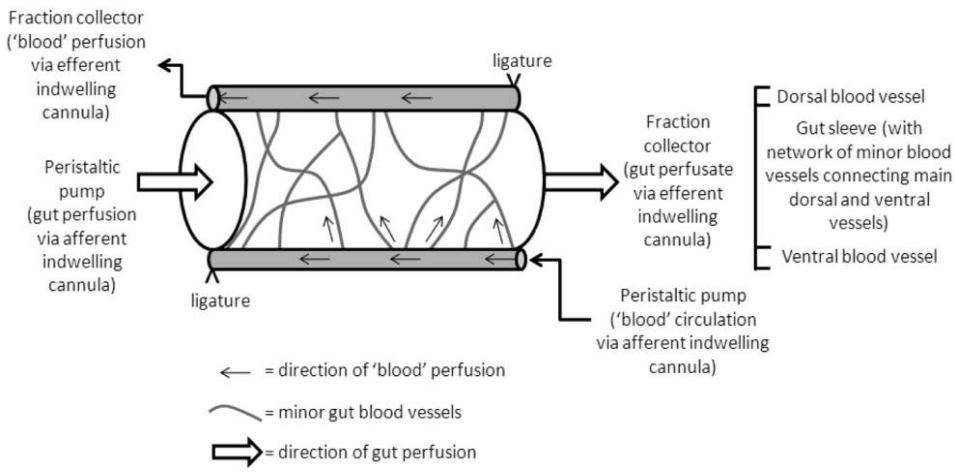

Figure 1. Diagrammatic representation of the double-perfused gut sleeve technique.

the major dorsal and ventral vessels, and then exit via the efferent cannula. Once cannulae were in place a 5–8-cm tract of intestine was excised, blood was flushed from the vessels using heparinized hagfish Ringer's solution (in mM: NaCl, 474; KCl, 8; $CaCl_2 \cdot 2H_2O$, 5; $MgSO_4 \cdot 7H_2O$, 3; $MgCl_2 \cdot 6H_2O$, 9; $NaH_2PO_4 \cdot H_2O$, 2.06; $NaHCO_3$, 41; and glucose, 5; pH = 7.6; Forster and Fenwick 1994) and the integrity of the blood circulation was tested (using food coloring dye). Gut cannulae (flared pieces of PE180 tubing; Intramedic [Clay Adams, Parsippany, NJ]) were then secured in place with surgical silk at both the anterior and the posterior ends of the excised intestinal section. The gut perfusate consisted of aerated hagfish Ringer's solution containing glucose that was perfused at a measured rate of 3.8 mL h^{-1}, countercurrent (anterior to posterior) to the direction of "blood" perfusate (hagfish Ringer's solution without glucose; perfusion rate, 4.0 mL h^{-1}). Perfusion was performed using a multichannel peristaltic pump (LKB Pharmacia, Uppsala). This technique allowed assessment of the glucose uptake, calculated as that which appeared in the perfusate. Perfused guts were placed in an aerated water bath (12°–15°C) and glucose absorption was assayed for a period of 2.5 h. Efferent gut and blood perfusates were collected for analysis of flow rates, and the latter was assessed for assessment of glucose concentration.

Glucose was assayed in the initial gut perfusates and in initial and final blood perfusate samples, using a commercial microplate assay (Biovision; Mountain View, CA). This kit utilizes enzymatic oxidation of glucose to generate a product that reacts with a dye to provide a color that, in association with known standards, allows for quantification of glucose via measurement of absorbance at 570 nm. The detection limit of the assay was 1 μM. Glucose uptake was expressed as a function of time and gut surface area, determined as described by Glover et al. (2003).

In Vitro Gut Sacs

For assessment of amino acid uptake, a standard gut sac technique was used (Glover et al. 2003). Briefly, sections of hagfish gut were excised and one end was closed with a surgical silk suture. A flared cannula was inserted in the other end and used for the introduction of filtered seawater (in mM: Na^+, 492; K^+, 9; Ca^{2+}, 12; Mg^{2+}, 50; Cl^-, 539; pH = 8.0) spiked with an appropriate concentration of "cold" amino acid (1 mM) and [^{3}H]-labeled amino acid (0.5–1 μC_i mL^{-1}; Perkin Elmer). The volume of filtered seawater introduced ranged from 0.3 to 2.4 mL, depending on gut sac size. The cannula was heat sealed and the gut sac was placed in a tissue bath (50-mL Falcon tube containing 10 mL of constantly aerated hagfish Ringer's solution). After 3 h, the suture was removed and the sac was flushed with cold displacement solution (100 mM glycine, 100 mM L-alanine). This removed the radio-labeled fluid and any adsorbed isotope from the sac. The mucosal surface (mucus and epithelial lining of the gut) was then scraped and excluded from analysis. These two steps ensured that isotope in the underlying gut tissue and in the serosal compartment represented amino acid that was truly absorbed. Surface area was then determined by applying a simplified calculation modeling the intestine as a simple tube (Glover et al. 2003), and radio-label accumulation in the gut tissue and serosal compartment were measured (see below). A previous study has shown that amino acid uptake characteristics do not change down the length of the hagfish intestine (Glover et al. 2011*b*), so the region of the gut from which the sac was sourced was not considered.

In Vitro Branchial Perfusion

Assessment of branchial glycine accumulation was made using a perfusion method (Glover et al. 2011*a*). Glycine was added to filtered seawater at a level of 50 μM and spiked with [^{3}H]-

glycine (1 μC_i mL^{-1}). This was constantly aerated and perfused for 3 h through medial gill pouches at a rate of ~6 mL h^{-1}. After the gill was flushed with cold displacement solution (see above) to remove unabsorbed radio-label solution, it was weighed subject to acid-digestion and assessment of radio-label accumulation (see below).

In Vitro Skin Flux

Skin from the medial dorsal region lateral to the gill apertures was removed and placed in a modified Ussing chamber for assessment of transmural (mucosal to serosal) amino acid flux (Glover et al. 2011*a*). Briefly, this method places skin across the aperture of a plastic scintillation vial, with the skin secured in place by the lid, which itself has an aperture. Radio-labeled amino acid ([^{3}H]-glycine or [^{3}H]-L-alanine; 0.5–1 μC_i mL^{-1}) spiked into filtered seawater on the mucosal side moves across the skin into hagfish Ringer's solution on the serosal side (inside the scintillation vial). Both mucosal and serosal solutions were constantly aerated throughout the assay. Amino acid concentrations of 50 μM were tested by spiking the mucosal solution. Following a cold displacement rinse (see above), the skin was acid-digested, and along with serosal samples it was assessed for isotope accumulation (see below).

Glycine Accumulation In Vivo during a 24-h Hypoxic Exposure

Individual juvenile hagfish (weight range, 15–50 g) were placed in 800-mL plastic exposure chambers with airtight lids. The seawater in each chamber was spiked with 10 μM glycine and 10 μC_i of [^{3}H]-glycine. Hagfish were allowed to gradually deplete the oxygen in the chambers, which resulted in water that was almost completely devoid of oxygen (<1 mmHg O_2) at the conclusion of the exposure period (24 h). As a control, normoxic hagfish were examined under identical conditions, with the exception that vessels were aerated throughout the exposure period.

After 24 h, hagfish were removed from exposure chambers and placed in a euthanasia/cold displacement solution containing 2 mg L^{-1} MS222 and 1 M glycine for up to 10 min. A blood sample was taken from the caudal sinus, and plasma and cell components were separated by centrifugation (2 min at 12,000 *g*). Dorsal skin sections were removed in a strip (from lateral to the eyespots, to the tail), and divided into anterior (eyespots to the first branchial aperture), medial (first branchial aperture to final branchial aperture), and posterior (final branchial aperture to tail) sections. The gills were removed and also separated in anterior (first three paired pouches), posterior (last three paired pouches), and medial (remaining pouches) sections. The intestine was excised and divided into anterior (first 2.5 cm), posterior (final 2.5 cm), and medial (remaining tissue) portions. Bile was removed from gall bladders, using a 23-gauge disposable needle, before the liver was removed. The heart and the tongue muscle were also dissected, along with ~1 g of skeletal muscle excised from the body wall adjacent to the gill apertures. Finally, the brain was removed. All tissues were weighed and acid-digested as described below before being counted via liquid scintillation.

Tissue Digestion and Radioisotope Analysis

All tissue samples were digested in appropriate volumes of 1 N HNO_3 and heated for 48–72 h in an oven at 60°C before digests (or subsamples up to a 2-mL volume) were added to 10 mL of Ultima Gold liquid scintillant (Perkin-Elmer, Boston, MA) for counting (Leonard et al. 2009). For aqueous samples, volumes of up to 1 mL were added to 4 mL of ACS liquid scintillant (GE Healthcare, Buckinghamshire). Counting was performed using a liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA) with quench correction based on the external standards ratio.

Data Analysis

Data were subjected to assessment of normality (Shapiro-Wilk or Kolmogorov-Smirnov tests) and equal variance (Levene's test) before being subjected to either parametric (*t*-test or one-way ANOVA) or nonparametric analysis (rank-sum test or Kruskal-Wallis ANOVA). Where ANOVA methods indicated significant differences, a Dunn's (nonparametric) or least significant difference (parametric) post-hoc test was applied. All statistical comparisons were performed at $\alpha = 0.05$, using Sigmaplot (ver. 11.2; Systat). Data are expressed as means ± SEM.

Results

Glucose

Exposure to hypoxic water resulted in reduced whole-blood oxygen levels. Blood Po_2 ranged from 4 to 15 mmHg relative to control levels of 37 to 55 mmHg. The P_{50} value for *Eptatretus stoutii* hemoglobin had previously been determined to lie in the range of 1–4 mmHg (Manwell 1958). Hypoxia significantly elevated plasma glucose levels from a normoxic level of 3.6 ± 0.7 to 9.1 ± 0.7 mmol L^{-1} after 24 h of exposure to hypoxia (Fig. 2). Controls were run, and these demonstrated that this effect was not a product of hagfish confinement (data not shown). Hagfish that had been exposed to hypoxia for 24 h did not, however, exhibit any significant increase in their capacity to absorb glucose from the digestive tract. Uptake of glucose in normoxic preexposed hagfish guts (113 ± 34 nmol cm^{-2} h^{-1}; $n = 6$) was statistically indistinguishable from that in hypoxic preexposed intestine (115 ± 33 nmol cm^{-2} h^{-1}; $n = 7$).

Glycine Uptake following Ammonia Preexposure

Preliminary studies demonstrated that Pacific hagfish were remarkably tolerant to ammonia and could withstand levels of 10 mM for periods of up to 96 h without any overt signs of toxicity. This was reflected in the skin and intestinal uptake results. No statistically significant changes in in vitro glycine uptake were observed in either gut (Fig. 3*a*) or skin (Fig. 3*b*;

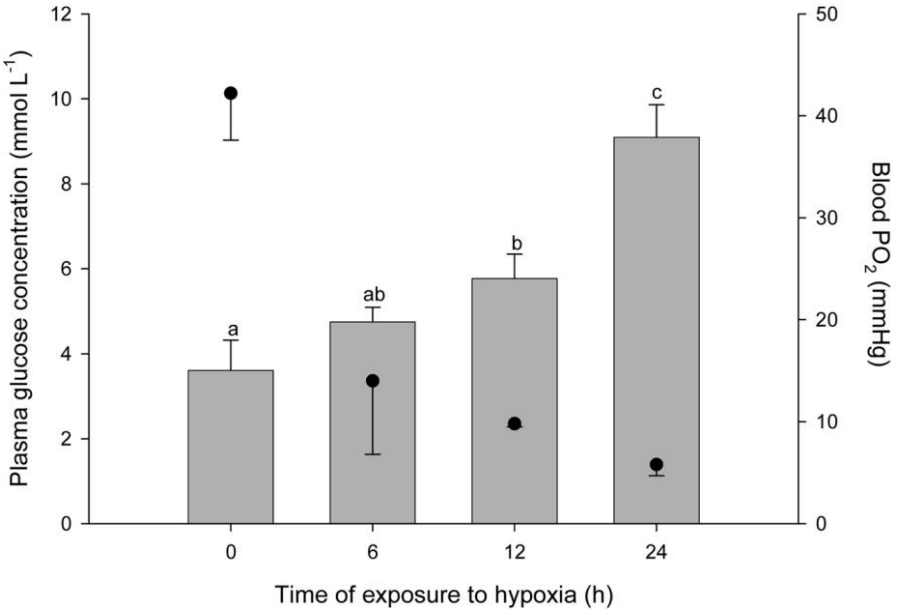

Figure 2. Time-dependent changes in blood Po_2 (mmHg; filled circles) and plasma glucose (mmol L^{-1}; gray bars) in Pacific hagfish exposed to hypoxia over the course of 24 h. Plotted values represent the means (±SEM) of six measurements at each time point. Bars sharing letters are not significantly different ($\alpha = 0.05$), as determined via one-way ANOVA followed by post hoc least significant difference analysis.

$P = 0.08$ for 500 μM) tissue collected from hagfish exposed to 10 mM ammonia for 24 h before uptake assessment, relative to hagfish that were not preexposed to ammonia.

L-Alanine and Glycine Uptake across Gill, Skin, and Gut Tissue

Tissue samples for transport assays were obtained from animals exposed to hypoxic waters for 24 h before tissue harvesting. Mean blood Po_2 levels of 7.6 ± 2.0 mmHg were determined. Glycine uptake in hypoxic hagfish gills was 10.9 ± 2.1 nmol g^{-1} h^{-1}, which represents a statistically significant threefold increase relative to normoxic gills (3.5 ± 0.9 nmol g^{-1} h^{-1}; Fig. 4). Conversely, glycine uptake across the skin was reduced relative to that of normoxic controls, falling from 1.5 ± 0.2 to 0.6 ± 0.1 nmol cm^{-2} h^{-1} (Fig. 4). An unchanged uptake of skin L-alanine uptake was observed.

The effects of 24 h of preexposure to hypoxia on in vitro intestinal L-alanine and glycine uptake are shown in Figure 5. Glycine uptake was stimulated sixfold by hypoxia, which is a highly significant increase over the normoxic control (Fig. 5*a*). In contrast, the uptake rate of L-alanine was unchanged by hypoxia exposure. However, the relative distribution of absorbed amino acid (as a percentage of total accumulated amino acid) was significantly altered for both L-alanine and glycine (Fig. 5*b*). Hypoxia led to a decreased proportion of amino acid accumulation in the gut tissue (and conversely, an increase in the proportion of amino acid that accumulated in the serosal compartment). Overall, however, the serosal component accounted for an average in excess of 95% of accumulated amino acid across all treatments.

Tissue Distribution of [^{3}H]-Glycine during Hypoxia

On the basis of the stimulation of glycine uptake in gill and gut following hypoxia exposure, it was hypothesized that glycine may have a critical role in facilitating hagfish function during periods of low environmental oxygen. To test this, hagfish were immersed for 24 h in hypoxic or normoxic water containing radio-labeled glycine. Hypoxia exposure resulted in a significant reduction in [^{3}H]-glycine accumulation in skin, plasma, main heart, liver, and tongue muscle tissue relative to animals subjected to normoxic water (Fig. 6*a*). The largest decrease was noted in the posterior skin section, where accumulation dropped from 12.7 ± 6.5 to 0.5 ± 0.1 μmol g^{-1}. Gut tissue also showed a decrease in accumulation (Fig. 6*a*) similar to that seen in the 3-h uptake assay (Fig. 5*b*), although this effect was not significant (anterior gut, $P = 0.132$; medial gut, $P = 0.065$; posterior gut, $P = 0.093$). Levels of glycine accumulation in the brain during normoxia were the highest of any tissue, at 67.5 ± 15.7 μmol g^{-1}, but during hypoxia these levels increased 5.7-fold to 380.6 ± 40.2 μmol g^{-1} (Fig. 6*b*). Skeletal muscle was included as a control, as it was not thought to be an active tissue in the confined exposure chambers; this tissue showed nearly identical levels of glycine accumulation between the hypoxic and normoxic exposures (9.0 ± 1.8 vs. 8.4 ± 2.8 μmol g^{-1}, respectively).

Discussion

Immersion in decaying carcasses is likely to expose hagfish to stressors, such as elevated ammonia levels and hypoxia, that have the potential to interfere with nutrient assimilation. This study indicates that uptake of glycine, L-alanine, and glucose

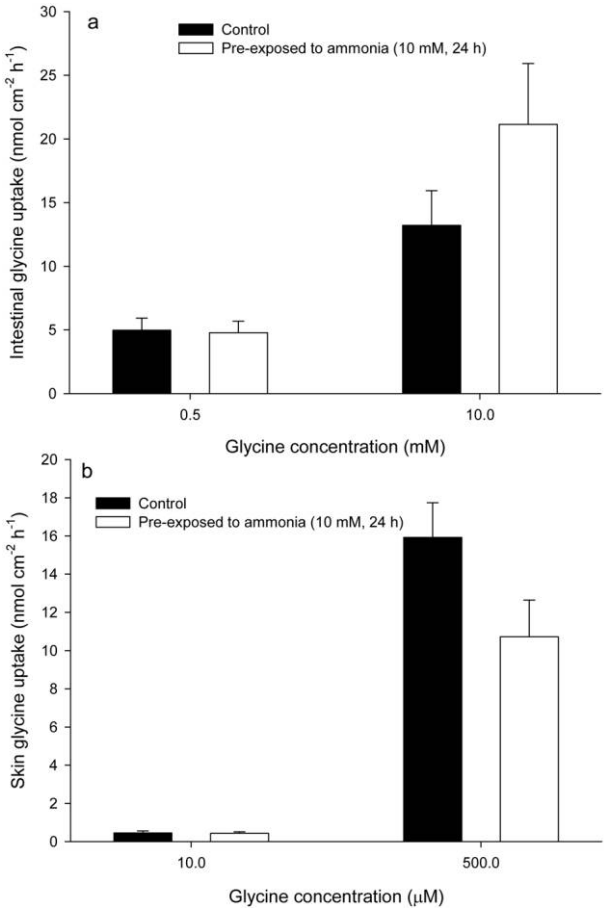

Figure 3. Uptake (nmol cm^{-2} h^{-1}) of [^{3}H]-glycine across intestine (*a*) and skin (*b*) of Pacific hagfish following 24 h of preexposure to waterborne ammonia (10 mM; white bars) or control seawater (black bars) as determined by in vitro transport assays. Plotted values represent the means (±SEM) of six preparations. No significant differences ($\alpha = 0.05$; one-way ANOVA) were noted.

is not compromised by preexposure to these stressors, and in fact the acquisition of nutrients such as glycine may be enhanced, presumably as a mechanism of protection against stressor-induced damage. These patterns of assimilation and metabolism may represent an adaptation to a spatially and temporally limited nutrient resource.

Effect of Hypoxia on Amino Acid Transport

Animals use two strategies to cope with low environmental oxygen levels (Hochachka and Lutz 2001). Hypometabolism involves the reduction of metabolically expensive processes, thus conserving ATP, while the second strategy results in maintenance of physiological functions, with an accompanying upregulation of ATP production via anaerobic metabolism (the Pasteur effect). With respect to absorptive processes, hagfish appear to adopt the latter strategy, which likely reflects their need to maximize nutrient acquisition. For example, L-alanine absorption was maintained at normoxic levels in all tissues examined. For the amino acid glycine, uptake from the dissolved nutrient pool (via the gill) and the diet (via the gut) was not only preserved but was actually significantly stimulated by hypoxia. Potential explanations for this effect are discussed below (see "Tissue Distribution of Glycine during Hypoxia"). Specific enhancement of glycine uptake suggests hypoxia-mediated regulation of a glycine transporter as opposed to an effect on the shared glycine/alanine transporter that appears to achieve the bulk of neutral amino acid absorption in hagfish gut and gill (Glover et al. 2011*a*, 2011*b*). Recently, mediators of hypoxic signaling (specifically, HIF2α) have been shown to stimulate iron absorption in mammalian intestine (Mastrogiannaki et al. 2009). This suggests that modulation of specific nutrient uptake pathways during hypoxia, as noted here for hagfish, may occur in other systems.

Studies that have examined the impact of hypoxia on nutrient transport in teleost fish are rare. In rainbow trout, a hypoxia-sensitive species, impacts of a mild reduction in oxygen levels had a variable impact on glycine uptake, with stimulation

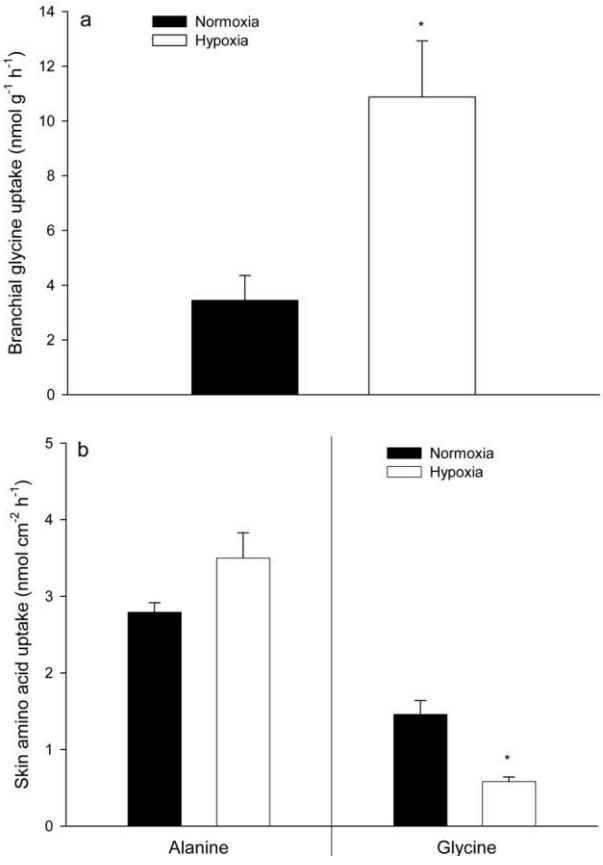

Figure 4. Branchial [^{3}H]-glycine uptake (at 50 μM; nmol g^{-1} h^{-1}; *a*) and skin uptake (at 50 μM; nmol cm^{-2} h^{-1}; *b*) of [^{3}H]-L-alanine and [^{3}H]-glycine in Pacific hagfish preexposed for 24 h to hypoxia (white bars) or normoxia (black bars) as determined by in vitro transport assays. Plotted values represent the means (±SEM) of five preparations. Statistically significant differences (indicated by asterisks) were determined at $\alpha = 0.05$, via *t*-test.

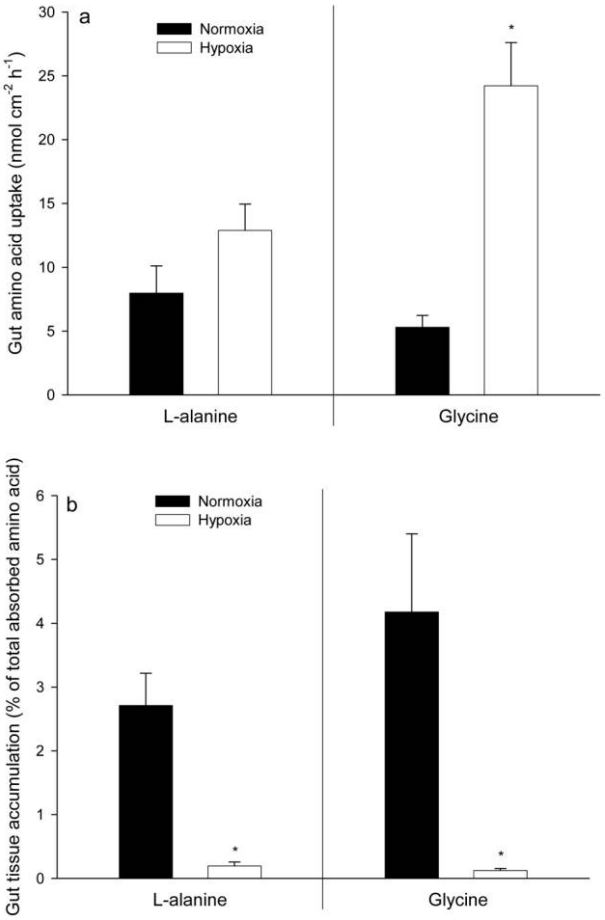

Figure 5. Effects of 24 h of preexposure to hypoxia (white bars) or normoxia (black bars) on intestinal uptake (*a*; nmol cm^{-2} h^{-1}) and gut tissue accumulation (*b*; % of total absorbed amino acid) of [^{3}H]-L-alanine and [^{3}H]-glycine (both at 1 mM) in Pacific hagfish as determined by in vitro transport assays. Plotted values represent the means (±SEM) of six preparations. Statistically significant differences (indicated by asterisks) were determined at $\alpha = 0.05$, via *t*-test (intestinal uptake) or Mann-Whitney *U*-test (proportional gut accumulation).

at low levels and inhibition at high levels (Boge et al. 1980). Severe hypoxia reduced sodium and fluid transport in the trout intestine but had no effect on copper absorption (Nadella et al. 2006). Overall, these effects in trout were either inhibitory or mildly stimulatory (increases on the order of 25%), compared with the large stimulatory response in hagfish gut, and they may be indicative of the relative tolerance of the two species to hypoxia.

For both L-alanine and glycine uptake across the gut, hypoxia caused a small but significant change in distribution of absorbed amino acid, with a decrease in tissue accumulation and a concomitant increase in serosal accumulation. This suggests a more rapid transfer of amino acid to the circulation and is consistent with a greater utilization of these substrates. The mechanism behind this effect is unclear. Hypoxia has been shown to reduce thickness in fish transport epithelia (Matey et al. 2008), which could facilitate altered distribution if this effect also occurs in the gut of hypoxic hagfish.

It is known that some aquatic invertebrates forced into hypoxic waters to feed delay digestive processing until they return to oxic waters (McGaw 2008). Under this scenario, the gastrointestinal tract would essentially function as a nutrient store. It is not known whether hagfish employ the same strategy. A period of feeding while immersed inside a hypoxic carcass followed by assimilation in a normoxic environment is replicated

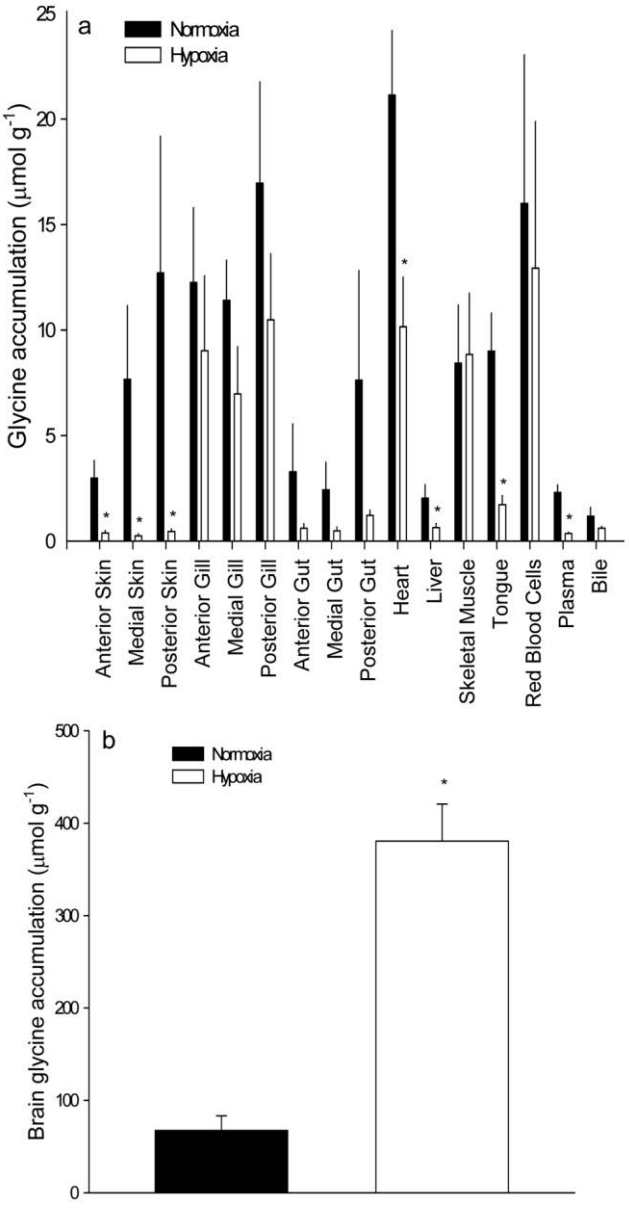

Figure 6. Accumulation (μmol g^{-1}) of [^{3}H]-glycine in body tissues (*a*) and brain (*b*) of Pacific hagfish following a 24-h in vivo exposure to hypoxia (white bars) or normoxia (black bars). Plotted values represent the means (±SEM) of six hagfish. Statistically significant differences (indicated by asterisks) were determined at $\alpha = 0.05$, via *t*-test or Mann-Whitney *U*-test.

by the experimental conditions employed during our in vitro assays for nutrient absorption. These transport assays were not conducted under hypoxic conditions because of concerns regarding tissue viability. The observed changes in glycine transport suggest that these tissues maintained a physiological "memory" of hypoxia and did not immediately return to a normoxic physiology once the hypoxic stimulus was removed.

It is important to consider that hagfish species differ in their tolerance to hypoxia (Forster 1998; Drazen et al. 2011). Therefore, the mechanisms displayed by *Eptatretus stoutii* in response to hypoxia may not be present in other species with less well-developed responses to decreased environmental oxygen.

Tissue Distribution of Glycine during Hypoxia

The changes in glycine uptake and distribution following 24 h of preexposure to hypoxia were suggestive of a critical role in hypoxia tolerance. To investigate this further, hagfish were immersed in a radio-labeled glycine solution to determine whether changes in the tissue distribution of glycine accumulation occurred during hypoxia. A significant decrease in glycine accumulation relative to normoxia was observed in epithelia and the tissues associated with feeding and nutrient distribution (liver, heart, tongue muscle, and plasma). Conversely, an increase in glycine levels in the brain was described. Levels of glycine are known to increase in the brains of anoxia-tolerant vertebrates such as carp and freshwater turtles (Nilsson 1990; Nilsson and Lutz 1991). As glycine facilitates postsynaptic hyperpolarization (Gundersen et al. 2005), an elevated glycine level reduces neural ATP use, thus inducing metabolic depression and conserving anaerobic energy reserves. The results of this study suggest that a similar mechanism occurs in an early vertebrate and also indicate that modifications to nutrient-transport properties can mobilize glycine from the diet in order to facilitate this response. The lack of a similar glycine accumulation in marine invertebrate neural tissue upon anoxia exposure (Reipschläger et al. 1997) suggests that this phenomenon may be a feature restricted to vertebrates. Given its phylogenetic placement, the hagfish may be a useful model in which to study the evolution of hypoxia tolerance in vertebrate animals.

Glycine is also known to have cytoprotective effects in mammalian systems, minimizing damage caused by hypoxic insult (Gundersen et al. 2005). A variety of mechanisms accounting for this protective effect have been proposed, including the modulation of apoptotic signaling (Jacob et al. 2003), stimulation of protective pathways (Nissim et al. 1992), and stabilization of membrane protein structure (Baines et al. 1990). This function may also have utility in the hypoxic hagfish brain.

Alanine is also a cytoprotectant in some systems (e.g., Estacion et al. 2003). While L-alanine uptake was unmodified by hypoxia preexposure in Pacific hagfish, an increase in serosal distribution of absorbed L-alanine was noted in gut sac experiments. If L-alanine does have a role in protection against hypoxia, this may be via mobilization from internal sources. Hagfish erythrocytes are known to accumulate high levels of L-alanine through a membrane transport system that exhibits very rapid transport rates (10,000 times those of mammals; Fincham et al. 1990). It has been proposed that this characteristic of red blood cell L-alanine metabolism may have a function in tissue amino acid supply (Fincham et al. 1990) and could thus contribute to cytoprotection during hypoxia. In the future, experiments utilizing an amino acid without known cytoprotective effects should be conducted to determine whether the altered distribution of L-alanine is related to a role in hypoxia defense or is simply a consequence of altered epithelial and tissue properties.

Glucose Metabolism

Carbohydrate metabolism in hagfish is generally considered to be more similar to that of mammals than of teleost fish and is believed to be related to their hypoxia tolerance (Young et al. 1994). Hagfish cardiovascular systems rely on glucose as a fuel during periods of lowered environmental oxygen levels (Forster 1998), and this is reflected by the graded increase in plasma glucose during hypoxia observed in this study. As this effect was not mediated by an enhanced gastrointestinal uptake of glucose, utilization of tissue glycogen stores is suggested. Glycogen stores in hagfish are found in the cardiac tissue (Helle and Lönning 1973), subdermal skin (Welsch et al. 1998), liver, and skeletal muscle (Emdin 1982), with the latter appearing to be the major storage site as indicated by concentrations more than twice those found in hepatic tissue (Emdin 1982). A recent study (Cox et al. 2010*b*) has shown that exposure of *E. stoutii* to anoxia leads to significant depletion of tissue glycogen, with an accompanying increase in blood and tissue lactate concentrations.

The increase in plasma glucose may also have an indirect role in metabolism. Glucose is known to increase the binding affinity of oxygen to hagfish hemoglobin via an effect related to altered water activity (Müller et al. 2003). Consequently, elevated plasma glucose levels may facilitate oxygen uptake in light of declining environmental availability.

No changes were found in the glucose transport characteristics of the gastrointestinal tract between animals that were preexposed to hypoxia and those exposed to normoxia, suggesting that the capacity for glucose uptake across the gut is maintained. This would allow the animal to continue to absorb nutrients such as glucose from the digestive tract despite the limitations that may be imposed by hypoxic feeding conditions. However, if in vivo responses to hypoxia, such as reduced vascular perfusion of the gut, occur in hagfish as they do in Atlantic cod (Axelsson and Fritsche 1991), then this may have an important role in reducing nutrient delivery. This requires further investigation. Our study suggests, however, that there are no sustained changes in glucose transport characteristics of gut tissue due to hypoxia. In this respect, hagfish gut tissue is distinct from mammalian gut tissue (Schoots et al. 2006), and this difference may be reflective of an adaptation to frequent hypoxia.

Glycine Uptake in Response to Ammonia Preexposure

Ammonia is known to impact epithelial permeability by modifying key enzymes responsible for epithelial transport (e.g., Abdoun et al. 2005; Alam and Frankel 2006) and may also cause histological damage to transport surfaces (e.g., Spencer et al. 2008). The finding of an unchanged glycine uptake in skin and gut following 24 h of exposure to elevated waterborne ammonia indicates that nutrient transport functionality is maintained during feeding in Pacific hagfish. This may be reflective of the remarkable ammonia tolerance of hagfish. Preliminary exposures showed 100% survival for extended periods (>96 h) at an excess of 10 mM of ammonium (167 μM as unionized ammonia). Among other marine fish, the intertidal-dwelling giant mudskipper (*Periophthalmodon schlosseri*) exhibits an acute median lethal concentration (96 h LC_{50}) of 120 mM (536 μM as unionized ammonia; Peng et al. 1998), while Gulf toadfish (*Opsanus beta*) display an ammonia LC_{50} of 9.75 mM (519 μM as unionized ammonia; Wang and Walsh 2000). The ammonia tolerance of hagfish is worthy of further investigation. Whether extended ammonia exposures and/or the presence of ammonia in the uptake assay medium impact nutrient transport was beyond the scope of this study.

Conclusion

The absorption of nutrients in hagfish can occur under significant environmental stress. Hypoxia and elevated ammonia levels are important stressors to most animals, but hagfish appear to be able to maintain transport properties of nutrients such as L-alanine and glucose despite the potential impacts of these conditions on basal physiological processes. Furthermore, hagfish have the ability to utilize their diets to promote tolerance, via the enhanced absorption of glycine. The exact role of glycine in hagfish hypoxia tolerance remains unknown, but by analogy with anoxia-tolerant species it may have neuromodulatory and/or cytoprotective actions.

The capacity for nutrient assimilation in conditions where oxygen availability is compromised has ramifications not only for the feeding environment but also for the tolerance of hagfish to other sources of hypoxia and anoxia. Hagfish are known to burrow in sediments that may become oxygen limited (Martini 1998), and for species such as *E. stoutii*, ecosystem change in the coastal regions of the north Pacific has resulted in an increased frequency of exposure to anoxic events (Chan et al. 2008). The results of this study suggest that *E. stoutii* has a capacity to withstand such environments without a short-term impact on critical processes such as assimilation of nutrients (at least those that have been studied thus far).

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