# Parental Males of the Plainfin Midshipman Are Physiologically Resilient to the Challenges of the Intertidal Zone

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## ABSTRACT

The decision of where to rear young is influenced by both the needs of offspring and the costs parents incur in certain rearing environments. Plainfin midshipman fish (*Porichthys notatus*) provide extended paternal care in rocky intertidal zones, where they experience regular bouts of aquatic hypoxia and air exposure during low-tide events. We investigated the physiological responses of plainfin midshipman males to three conditions for 6 h that simulate what these fish naturally experience during tidal cycles while nesting: normoxia, progressive hypoxia, or air exposure. Hypoxia- and air-exposed fish exhibited shifts in energy metabolites, driven largely by elevated lactate and glucose content and reduced glycogen content in several tissues (muscle, heart, liver, and brain), but the magnitude of these changes was relatively modest. Hematocrit increased most in air-exposed fish relative to normoxia-exposed fish, contributing to an increase in whole-blood hemoglobin concentration. Air exposure reduced swim bladder oxygen content, suggesting that internal $O_2$ stores are drawn on during air exposure. In a second experiment, we found that aquatic surface respiration and gill ventilation frequency increased in hypoxia-exposed fish relative to normoxia-exposed fish. Overall, our results suggest that plainfin midshipman overcome the challenges of the intertidal environment through a variety of physiological strategies and exhibit little physiological disturbance in response to the fluctuating and extreme conditions created by regular low tides.

*Keywords:* air exposure, hypoxia, intertidal zone, ecophysiology, nest site selection, anaerobic respiration.

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## Introduction

Parental care enhances offspring fitness but often comes at a cost to the future reproductive success of parents (Trivers 1972; Fromhage 2017). Parental care commonly occurs after eggs are fertilized or laid but can also take place prenatally through the careful selection and maintenance of a nesting or rearing site. Acquiring and maintaining nesting or rearing sites that maximize offspring growth and survival can impose significant costs to parents, diminishing their future reproductive potential. For example, mother water pythons (*Liasis fuscus*) that brood their young in relatively cool sites reduce predation pressure on their offspring but must guard for longer and do so without feeding, thus suffering body condition deterioration (Madsen and Shine 1999). Similarly, female painted turtles (*Chrysemys picta*) that leave the water to deposit their eggs in sites with low canopy cover, where offspring are more likely to survive, endure physiologically costly lactate and temperature increases as a result of this strenuous activity (Congdon and Gatten 1989; Hughes and Brooks 2006; Pruett et al. 2019). Thus, a parent's decision to nest at a particular location might benefit offspring in the short term but can also expose a parent to environmental challenges that diminish its safety and body condition.

Some nesting habitats are so environmentally challenging that parents typically do not remain there to provide posthatching care. The intertidal zone represents such an environment for many fishes, as the majority that breed there (known as beach-spawning fishes) simply abandon their eggs after they hatch (Coleman 1999). The intertidal habitat can provide numerous benefits to developing fish embryos, including higher $O_2$ availability (Strathmann and Hess 1999), warmer temperatures (Bose et al. 2019; N. Brown, N. Houpt, N. Yee, J. Curtis, B. Bolker, F. Juanes, and S. Balshine, unpublished manuscript), and reduced aquatic predation (Tewksbury and Conover 1987). These conditions can enhance

offspring development rate and survival (Tewksbury and Conover 1987; Smyder and Martin 2002). However, the intertidal zone also exposes caregiving parents to environmental challenges when the tides recede, including aquatic hypoxia in isolated tide pools and air emersion (Richards 2011). For water-breathing fishes, these conditions impose a wide range of physiological challenges (Burnett 1997; Richards 2009, 2011), which might explain why so few fishes remain in the intertidal zone to provide posthatching care.

Aquatic hypoxia is common in intertidal waters and can challenge the ability of organisms to maintain sufficient rates of $O_2$ supply to support cellular $O_2$ demands (Hochachka et al. 1996; Boutilier 2001; Bickler and Buck 2007; Richards 2011). Accordingly, organisms that encounter environmental hypoxia can respond with a variety of behavioral or physiological responses to avoid or mitigate its effects. For example, many fishes detect and preferentially avoid hypoxic water when possible (Kramer 1987; Wannamaker and Rice 2000; Chapman and McKenzie 2009) or initiate aquatic surface respiration (ASR) to take advantage of the relatively $O_2$-rich water at the air-water interface (Kramer and McClure 1982; Wannamaker and Rice 2000; Dixon et al. 2017). When behavioral avoidance of environmental hypoxia is impossible or insufficient, a number of physiological responses can buffer its harmful effects. Many fishes increase $O_2$ uptake during hypoxia by increasing gill ventilation frequency (Hughes 1973; Nikinmaa and Rees 2004; Scott et al. 2017) and/or reversibly remodeling their gill morphology to increase surface area for gas exchange (Sollid et al. 2003; Matey et al. 2008; Wu et al. 2017). Hypoxia-exposed fish often maintain blood $O_2$ content by increasing hemoglobin (Hb) concentration, resulting from erythropoiesis, and/or increasing Hb–$O_2$ binding affinity by reducing the concentration of allosteric modulators of Hb–$O_2$ binding in red blood cells (Nikinmaa and Rees 2004; Tervonen et al. 2006; Richards 2011). Certain fishes have also been suggested to draw on the $O_2$ stores in their swim bladders during hypoxia (Berg and Steen 1965; Todd and Ebeling 1966). The use of anaerobic glycolysis often increases during hypoxia to help maintain adenosine triphosphate (ATP) supply despite decreased $O_2$ availability, and this is normally associated with increases in tissue lactate levels and reductions in tissue glycogen content and pH (Milligan and Wood 1986; Gracey et al. 2001; Speers-Roesch et al. 2013; Borowiec et al. 2015, 2018). Some hypoxia-tolerant fishes can also depress their metabolic rate in $O_2$-limited conditions to reduce $O_2$ demands (Muusze et al. 1998; Richards 2009). In some species, air exposure may present similar challenges to those experienced during aquatic hypoxia and thereby lead to similar physiological responses, but some species (including several intertidal species) can help avoid cellular $O_2$ limitation through aerial gas exchange using the skin or gills (Graham 1999; Martin et al. 2004; Wright 2012). These strategies are not mutually exclusive, and fishes may favor certain strategies over others as a result of interspecific differences and variation in the severity, duration, or pattern of hypoxia or air exposure they normally experience (Sloman et al. 2008; Mandic et al. 2009; Richards 2011; Borowiec et al. 2015, 2018). For example, intertidal resident fishes, which encounter regular bouts of hypoxia and air exposure throughout the tidal cycle, typically display a range of strategies for coping with these conditions, whereas subtidal species are typically less tolerant (Martin 1996; Sloman et al. 2008; Mandic et al. 2009).

Despite the physiological challenges encountered by intertidal fishes, there is at least one family of normally subtidal fish that provides extended parental care (60 d of care, including posthatching care) in this dynamic environment: the toadfishes (Batrachoididae; Arora 1948; Coleman 1999). The plainfin midshipman (*Porichthys notatus*) is a well-studied marine toadfish that is resident to the subtidal but migrates from depths exceeding 200 m to rocky intertidal zones along the Pacific coast of North America for breeding (Arora 1948; Sisneros 2004). Once in the intertidal zone, guarder males (also called parental or type I males) excavate, defend, and attract gravid females to their nesting cavities underneath large rocks, which are built at varying intertidal elevations (Brantley and Bass 1994). After obtaining broods of eggs, guarder males remain in their nests, cleaning and protecting their young from predators, for an extraordinarily long period of up to 2 mo, until the juveniles become free swimming (Cogliati et al. 2013).

Nest site selection in plainfin midshipman has been hypothesized to depend on a trade-off between choosing an elevation that is favorable for developing embryos and choosing a tolerable place for caregiving adults to remain for several months (Bose et al. 2019). As nesting elevation increases, abiotic conditions and fluctuations that might be favorable to offspring development but that may also prove to be challenging for adults become more severe (e.g., temperature changes and duration of air emersion at low tide increases in magnitude with elevation; Bose et al. 2019). However, neither the developmental benefits of nesting elevation to young nor the physiological costs to parents have been thoroughly investigated. In this study, we focused on a key prediction of the trade-off hypothesis, namely, that paternally caring plainfin midshipman incur physiological costs in response to the environmental conditions they experience during care. The physiological challenges of intertidal nesting include frequent exposure to aquatic hypoxia and air exposure when nesting cavities are isolated from the surrounding ocean at low tide, which occurs for longer durations at higher elevations (Bose et al. 2019). Previous research suggests that these conditions impose some physiological costs to plainfin midshipman guarder males. For example, guarder males experience increased plasma lactate content and decreased glycogen content in the liver and brain in their nests during low tide (Bose et al. 2019). Additionally, males exposed to aquatic hypoxia in controlled laboratory settings exhibit elevated plasma and heart lactate content (Craig et al. 2014). While these results give some insight into the responses of plainfin midshipman guarder males to intertidal conditions, their interpretation is limited because they lack a controlled comparison of the responses of these fish to the full range of conditions experienced in the wild (normoxia, hypoxia, and air exposure). Given that air exposure is a common stressor for plainfin midshipman at low tide and that some fishes respond differently to air exposure than aquatic hypoxia (Turko et al. 2014), such a comparison is needed to elucidate the magnitude of physiological costs that guarder males endure in their nesting environment. Our goal was to discern these costs in order to refine our understanding of the parental sacrifice incurred

by plainfin midshipman during their uniquely prolonged parental care period in the intertidal zone. Additionally, we sought to describe the coping mechanisms that guarder males exhibit in response to aquatic hypoxia and air exposure that might mitigate the daily physiological costs of occupying the intertidal zone and thus facilitate the prolonged parental care period in this species.

To determine how plainfin midshipman respond to environmental conditions in the intertidal zone, we exposed guarder males to normoxia, aquatic hypoxia, or air emersion to mimic common intertidal abiotic variation. We surveyed key energy metabolites (lactate, glucose, and glycogen) and intracellular pH in a variety of tissues, as well as indexes of $O_2$ transport and internal $O_2$ stores (hematocrit, whole-blood and mean cell Hb concentration, and swim bladder $O_2$ content) after a 6-h exposure to simulated low-tide conditions. In a second experiment, we measured the frequencies of gill ventilation and ASR in normoxia and aquatic hypoxia to investigate respiratory responses to low-tide conditions.

## Methods

### *Field Collection of Animals*

Fifty-seven guarder males were collected from Ladysmith Inlet (48°59′43″N, 123°48′57″), Bowser Lagoon (49°26′20″N, 124°41′01″E), and Lantzville Beach (49°09′57″N, 123°56′24″E), British Columbia, between May and July 2018 by gently overturning large rocks to uncover nesting cavities underneath. Males were placed by hand in 40-L aerated bins filled with seawater and were transported for 2–3 h to the University of Victoria Outdoor Aquatic Facility. All animals survived transport and were housed outdoors in aerated 477-L cylindrical acrylic stock tanks (≤8 fish per tank) supplied with ~13°C flow-through seawater and enriched with pea gravel and brick shelters. Guarder males were not fed while being housed, consistent with their limited access to food during nesting in the wild (Sisneros et al. 2009; Bose et al. 2014; Cogliati et al. 2015). Fish were collected over 1–2 d during the lowest tide in the spring cycle and were used in experiments during the subsequent ≤10 d until the next low-tide period. All fish were given a minimum of 24 h to recover before undergoing a trial.

### *Experiment 1: Physiological Responses to Hypoxia and Air Exposure*

*Exposure Protocol.* Each day, from the stock tanks we selected three plainfin midshipman guarder males that were similar in body mass (mean difference of 6% body mass between the largest and smallest fish run on a given day). We placed each fish in a separate covered 15-L plastic bin containing a 2-cm layer of pea gravel as substrate. Lids contained a hole through which a flexible pipe was inserted, bringing a continuous flow of ~13°C seawater. Fish were left to adjust to these conditions overnight. The volume and space provided by these bins reflect the confinement observed in natural plainfin midshipman nests found in the wild (Bose et al. 2018). Summary information on measurements and condition of fish in each treatment are provided in table A1.

The following morning, each individual received one of three treatments assigned in a randomized order. In the normoxia (control) treatment, fish remained in well-oxygenated seawater (>9 mg $L^{-1}$); these conditions were identical to the overnight adjustment period (fig. 1*a*). In the hypoxia treatment, water inflow was halted, and water was siphoned out until only enough water to cover half of the fish's operculum remained (~14 mL of water per gram of fish; fig. 1*b*). As a result, hypoxia became progressively more severe over time as the fish consumed the $O_2$ in the water. In the air exposure treatment, water inflow was halted, and all seawater was siphoned out, leaving the fish moist but fully air exposed (fig. 1*c*). Each treatment lasted for 6 h, reflecting the duration that plainfin midshipman nests become separated from the surrounding ocean during a long low-tide event (Bose et al. 2019). To control for <3 min of disturbance associated with beginning hypoxia and air exposure treatments, we subjected fish in the control treatment to sham disturbances (lifting tank lid, adjusting water inflow pipe, inserting siphon tube, etc.), but we did not siphon any water, and the water inflow remained constant. All fish were misted with seawater every 1 h through a small hole in the bin lid to ensure that they did not desiccate. This was necessary because air-exposed fish in the field are sometimes able to burrow into moist substrate to mitigate desiccation, but this was not possible in the exposure bins. We measured temperature (±0.1°C; Oakton RDO 450), dissolved oxygen (DO; ±0.1 mg $L^{-1}$; Oakton RDO 450), and ammonia concentration (±0.5 ppm; Aquachek Ammonia Test Strips) at the beginning and end of the overnight adjustment period and again at the end of each 6-h exposure. Temperature and DO did not change during the 6-h exposure in the normoxia treatment (temperature: *t*-test, $t = -0.587$, df = 25.58, $P = 0.56$; DO: *t*-test, $t = 1.716$, df = 19, $P > 0.10$; table A1), while temperature increased and DO decreased during the course of the hypoxia treatment (temperature: *t*-test, $t = -3.946$, df = 15.52, $P = 0.001$; DO: *t*-test, $t = 8.938$, df = 14.16, $P < 0.001$; table A1). Final temperature was highest in the air exposure treatment, reaching an average of 16.7°C (ANOVA, $F = 57.99$, df = 2, 24 $P < 0.001$; table A1). Similar DO levels and temperatures have been recorded in plainfin midshipman nests in the field during spring low tides (Bose et al. 2019). We ran one fish per treatment each day for 15 d, resulting in a sample size of 45 fish ($N = 15$ fish per treatment).

*Dissection and Tissue Collection.* Following the 6-h exposures, fish were euthanized quickly via an overdose of benzocaine (1 g $L^{-1}$ benzocaine–seawater bath). Once unresponsive to tactile stimulation of the caudal fin, we collected blood in heparinized capillary tubes from the caudal artery via caudal severance. Blood was transferred to 1.5-mL Eppendorf tubes and centrifuged at 2,000 rpm for 1–2 min to separate plasma and red blood cells (RBCs); both were then frozen in liquid nitrogen in separate tubes. Some whole blood was also collected in microcapillary tubes that were then centrifuged for 10 min at 6,900 rpm to measure hematocrit.

Following blood collection, we extracted a transverse steak of posterior tail muscle, a liver tissue sample, and the whole heart

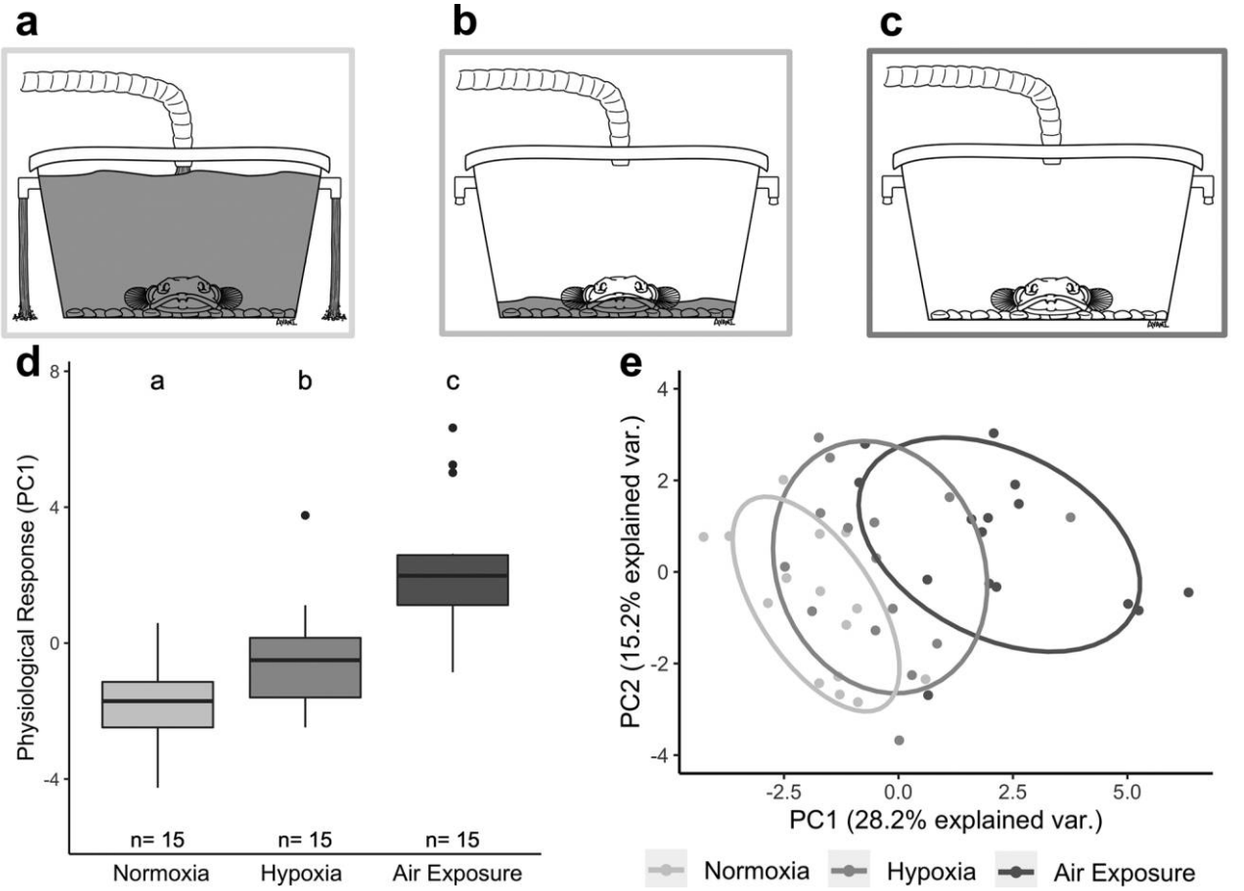


Figure 1. Illustrations of the normoxia (*a*), hypoxia (*b*), and air exposure (*c*) treatments from experiment 1. *d*, Physiological response of fish exposed to simulated tide conditions as captured by principal component 1 (PC1). *e*, Principal component analysis biplot grouped by treatment. Increasing PC1 was associated with increasing glucose and lactate content as well as decreasing intracellular pH ($pH_i$) and glycogen content in plasma and tissues. Increasing principal component 2 (PC2) values were associated with increasing $pH_i$. Different letters above each boxplot denote significant differences at the level of $P < 0.05$ from post hoc Tukey tests.

and brain; all tissues were freeze clamped and placed in liquid nitrogen. The swim bladder was dissected and weighed ($\pm 0.01$ g; Acculab VIC-612), and its volume ($V_S$ in mL) was quickly measured using standard volumetric methods (as described in Scherle 1970). A retractable needle-type fiber-optic oxygen sensor (Pyroscience Firesting $O_2$) was inserted into the left lobe of the swim bladder to record partial $O_2$ pressure ($P_{O_2,S}$ in Torr). Swim bladder $O_2$ content was calculated as $(V_S \times P_{O_2,S}) \cdot (P_B)^{-1}$, where $P_B$ represents barometric pressure (in Torr) of the atmosphere.

*Intracellular pH and Metabolite Assays.* Frozen muscle, heart, liver, and brain tissues were ground into a fine powder using a mortar and pestle cooled with liquid nitrogen and were stored at −80°C until assayed.

Intracellular pH ($pH_i$) was measured in powdered tissues and in frozen RBCs using methods previously described in Pörtner (1990), Pörtner et al. (1990), Baker et al. (2009), and Borowiec et al. (2018). Powdered muscle, heart, liver, and brain tissues (10–100 mg) were briefly homogenized in 0.5-mL microtubes with 200 μL of an ice-cold homogenization solution (6 mmol $L^{-1}$ nitrilotriacetic acid, 150 mmol $L^{-1}$ potassium fluoride). Tubes were quickly capped to prevent escape of carbon dioxide and incubated on ice for 10 min. RBCs (20–100 μL) were lysed by three cycles of freeze-thaw; 5 μL was removed from that lysate for later determination of Hb concentration (using Drabkin's reagent, following manufacturer's instructions; Sigma-Aldrich, Oakville, Ontario). The lysed cells were diluted into 100 μL of ice-cold homogenization solution and then incubated on ice for 10 min. Following incubation, homogenized tissues and RBCs were vortexed to ensure thorough mixing, and $pH_i$ was measured using a glass microelectrode preconditioned with ice-cold homogenization solution (Sartorius, Bohemia, NY) within 20 s.

Methodologies for quantifying plasma and tissue lactate were adapted from Borowiec et al. (2018). Tissue samples (~25 mg) were homogenized in 300 μL of 6% ice-cold $HClO_4$ using a PowerGen 125 electric homogenizer on its highest setting for 20 s (Fisher Scientific, Whitby, Ontario). Plasma samples (~40 μL) were diluted in 80 μL of 6% $HClO_4$. Aliquots of tissue (100 μL) and plasma (40 μL) homogenate were set aside for later determination of glucose and glycogen levels. The remaining samples were

neutralized with 3 mol $L^{-1}$ $K_2HCO_3$ ($6.8 \leq pH \leq 7.2$) and centrifuged at 4°C and 10,000 rpm for 10 min. The resulting supernatant was used to measure lactate content by standard methods adapted for a 96-well plate (Bergmeyer 1983) with initial lactate assay conditions as follows: 2.5 mmol $L^{-1}$ $NAD^+$ in glycine buffer (0.6 mol $L^{-1}$ glycine, 0.5 mol $L^{-1}$ hydrazine sulphate, pH 9.4). Assays began by adding an excess amount (5 U $mL^{-1}$) of lactate dehydrogenase (LDH). Lactate was quantified in each sample based on appearance of NADH in the well following the addition of LDH using a lactate standard curve. The lactate assay was run in triplicate at 37°C on a SpectraMax 384 microplate reader (Molecular Devices, Sunnyvale, CA).

To measure free glucose and glycogen (powdered tissues only), we added $K_2HCO_3$ and acetate buffer (pH = 4.8; Sigma-Aldrich) to the reserved acidified homogenates, both to final concentrations of 167 mmol $L^{-1}$. Tissue samples were then split into two equal aliquots. The first aliquot was used to determine total glucose content by digesting the glycogen in the solution with 4 U $L^{-1}$ amyloglucosidase (suspended in 300 mmol $L^{-1}$ Tris-HCl, 4.05 mmol $L^{-1}$ $MgSO_4$, pH = 7.5) for 2 h at 40°C. The second aliquot was used to determine free glucose content and was incubated without amyloglucosidase at 4°C for 2 h. Samples were then neutralized with 3 mol $L^{-1}$ $K_2HCO_3$ ($6.8 \leq pH \leq 7.2$) and then centrifuged at 4°C and 10,000 rpm for 10 min. Glucose concentration was determined by measuring the appearance of NADPH in the samples following the addition of an excess of the coupling enzyme hexokinase (5 U $mL^{-1}$) using a standard curve. Glucose was measured in duplicate under the following conditions: 1 mmol $L^{-1}$ ATP, 0.5 mmol $L^{-1}$ $NADP^+$, and 5 mmol $L^{-1}$ $MgCl_2$ in 20 mmol $L^{-1}$ imidazole (pH = 7.4) with excess coupling enzyme (5 U $mL^{-1}$ glucose-6-phosphate dehydrogenase). The difference in free glucose content between the digested and undigested tissue aliquots was used to calculate glycogen content.

### *Experiment 2: Respiratory Response to Hypoxia*

*Exposure Protocol and Data Collection.* We used 15-L plastic bins similar to those used in experiment 1, but in this case they contained a plastic ramp (21° angle incline) that extended the full length of each bin, providing a platform on which fish could emerge fully or partially during the trial (methodology adapted from Sloman et al. 2008). Guarder males were held overnight with flow-through 13°C seawater as in experiment 1. After the adjustment period, fish were exposed to either normoxia or hypoxia for 8 h. In the normoxia condition, the inflow of water to the bin was removed, the water was drained to the top of the plastic ramp, and a bubbling air stone was inserted to prevent DO from decreasing (fig. 3*a*). In the hypoxia condition, the water inflow was removed, the water was drained to just above the head of the fish (covering only a small portion of the bottom of the ramp), and an inactive air stone (i.e., no air bubbling through) was inserted (fig. 3*b*). Thus, the two treatments had different water depths within the exposure bins, but fish had the opportunity to perform ASR or air emergence or remain below the water's surface in both treatments. In the normoxia condition, DO decreased slightly over the first hour and then remained steady at ~8.4 mg $L^{-1}$ (fig. A1). DO in the hypoxia treatment decreased over time, eventually settling at ~1.5 mg $L^{-1}$ after 6 h (fig. A1). DO was significantly lower in the hypoxia treatment at all time points beyond initial levels at the start of each trial (*t*-tests, $P < 0.001$). Twelve guarder males were used in experiment 2, and each was tested in both treatments (24 h between trials), counterbalancing the order. Summary information on measurements and condition of fish used in experiment 2 is provided in table A2.

Following the onset of each exposure, the position on the ramp and respiration behavior (submerged, surface, or air respiration) of each fish were recorded every 20 min. DO was recorded every hour, or whenever a fish exhibited a marked change in respiratory behavior (e.g., initiating ASR), through a hole in the lid of each bin. We took care to avoid disturbing the fish during these measurements. Every 2 h, gill ventilation frequency was estimated by counting the number of operculum movements over a 2-min period (counts were taken by observation through a small hole in the lid of the exposure bin).

### *Statistical Analyses*

All analyses were performed using the statistical software R (ver. 3.5.2; R Development Core Team 2018). We used a principal component analysis (PCA) to evaluate the overall variation in plasma and tissue metabolites in response to normoxia, hypoxia, or air exposure. Missing values in the metabolite data set (e.g., due to insufficient tissue; of 855 possible data points, 94 [11%] were missing) were filled using single imputation (predictive mean matching with five iterations; *mice* package in R; van Buuren and Groothuis-Oudshoorn 2011). Results were qualitatively similar when fish with some missing metabolite values were excluded from the analysis.

All metabolite variables were $\log_{10}(x + 1)$ transformed, mean centered, and standardized by dividing by their standard deviations before entering the PCA (Schielzeth 2010). Principal component 1 (PC1; 28.2% of variation explained) and 2 (PC2; 15.2% of variation explained) loadings are summarized in table 1, and a biplot of these components is shown in figure A2. PC1 increased as $pH_i$ (indicative of metabolic acidosis) and glycogen (an energy storage molecule) decreased and as lactate (the end product of anaerobic glycolysis) and free glucose increased. We therefore considered PC1 a measure of the physiological response in each fish. PC2 increased as $pH_i$ increased. We tested whether physiological responses varied with treatment by fitting a linear mixed effects model (LMM) to PC1. Treatment group (three-level categorical variable: normoxia, hypoxia, or air exposure) was included as a predictor variable, and trial date (as a factor) was included as a random intercept. Julian date of trial was also included as a continuous predictor variable to determine whether the responses of fish to treatment conditions varied over the duration of experiment 1. We then examined differences between treatment groups by using Tukey's pairwise contrasts. We used similar statistical models to determine the effect of treatment group on PC2. Similar models, but without Julian date included as a continuous predictor, were also fitted with hematocrit and whole-blood

Table 1: Summary of plasma and tissue metabolite content and principal component 1 (PC1) and 2 (PC2) loadings from experiment 1

| Metabolites | Normoxia exposed | Hypoxia exposed | Air exposed | PC1 loadings | PC2 loadings |
|---|---|---|---|---|---|
| Lactate: | | | | | |
| Plasma | .11 ± .32$^{A}$ | .43 ± .64$^{AB}$ | 1.36 ± .92$^{B}$ | .330 | .221 |
| Muscle | .96 ± .42$^{A}$ | 1.42 ± .56$^{B}$ | 1.64 ± .56$^{B}$ | .293 | .150 |
| Heart | 1.61 ± .66$^{A}$ | 1.70 ± .66$^{A}$ | 2.21 ± .68$^{A}$ | .248 | −.113 |
| Liver | 1.58 ± .73$^{A}$ | 1.83 ± .55$^{A}$ | 2.01 ± .46$^{A}$ | .111 | .250 |
| Brain | 1.14 ± .43$^{A}$ | 1.22 ± .64$^{A}$ | 1.59 ± .70$^{A}$ | .151 | .212 |
| $pH_i$: | | | | | |
| Muscle | 7.50 ± .07$^{A}$ | 7.49 ± .08$^{A}$ | 7.45 ± .09$^{A}$ | −.240 | .338 |
| Heart | 7.39 ± .15$^{A}$ | 7.40 ± .16$^{A}$ | 7.37 ± .17$^{A}$ | −.169 | .438 |
| Liver | 7.31 ± .13$^{A}$ | 7.34 ± .12$^{A}$ | 7.28 ± .11$^{A}$ | −.213 | .380 |
| Brain | 7.41 ± .12$^{A}$ | 7.43 ± .13$^{A}$ | 7.47 ± .12$^{A}$ | −.064 | .425 |
| RBC | 7.15 ± .20$^{A}$ | 7.21 ± .13$^{A}$ | 7.04 ± .23$^{A}$ | −.165 | −.100 |
| Glucose: | | | | | |
| Plasma | 1.19 ± .38$^{A}$ | 2.03 ± .67$^{B}$ | 3.53 ± 1.61$^{C}$ | .402 | .101 |
| Muscle | .37 ± .10$^{AB}$ | .34 ± .21$^{A}$ | .54 ± .22$^{B}$ | .040 | −.130 |
| Heart | 1.16 ± .51$^{A}$ | 2.02 ± 1.06$^{B}$ | 3.13 ± 1.27$^{C}$ | .320 | .151 |
| Liver | 2.00 ± 3.30$^{A}$ | 3.07 ± 3.67$^{A}$ | 8.09 ± 3.91$^{B}$ | .336 | .189 |
| Brain | .80 ± .52$^{A}$ | 1.40 ± .50$^{A}$ | 2.92 ± 1.41$^{B}$ | .337 | −.104 |
| Glycogen: | | | | | |
| Muscle | 4.67 ± 2.49$^{A}$ | 4.55 ± 2.17$^{AB}$ | 2.79 ± 1.50$^{B}$ | −.198 | .176 |
| Heart | 20.50 ± 5.76$^{A}$ | 19.30 ± 5.51$^{A}$ | 15.79 ± 5.30$^{A}$ | −.063 | −.090 |
| Liver | 129.07 ± 36.6$^{A}$ | 120.91 ± 38.3$^{A}$ | 112.50 ± 44.8$^{A}$ | −.090 | −.178 |
| Brain | 9.39 ± 2.34$^{A}$ | 9.31 ± 2.70$^{A}$ | 9.57 ± 4.49$^{A}$ | −.040 | .005 |
| Variation explained (%) | | | | 28.2 | 15.2 |

Note. Plasma and tissue metabolites are given in millimoles per liter (mmol $L^{-1}$) and micromoles per gram wet mass ($\mu$mol $g^{-1}$ wet mass), respectively. Mean ± SD shown for each metabolite. Linear mixed effect models were fitted to each endpoint, with trial date added as a random intercept. Tukey tests were used to examine differences between pairs of treatment groups, and the results of these pairwise tests are shown in table A3. Significant differences between treatment groups at the level of $P < 0.05$ from post hoc Tukey tests are denoted by different superscript capital letters next to each mean metabolite value.

and mean cell Hb content as continuous response variables. Similar models were also used to determine the effect of treatment on swim bladder $O_2$ content and swim bladder volume but with fish mass added as a covariate. Swim bladder volume and $O_2$ content were standardized to fish mass for graphical representation but not for statistical analysis.

To determine the effect of DO content on gill ventilation frequency (count $min^{-1}$) in experiment 2, we fitted an LMM with DO as a continuous predictor variable and fish ID as a random intercept. We also included trial date (as a factor) as a random intercept to account for variation among experimental days. DO and gill ventilation frequency were both log transformed to satisfy parametric assumptions. A generalized linear mixed effects model (GLMM) was fitted to test whether the proportion of total time points in which fish performed ASR was different under normoxia versus hypoxia treatments. Treatment group (two-level categorical variable: normoxia or hypoxia) was included as a predictor variable. Fish ID and trial date (as a factor) were added as random intercepts.

### *Ethical Note*

Plainfin midshipman fish are an abundant marine species that have been placed in the category of "Least Concern" by the International Union for the Conservation of Nature (Collette et al. 2010). All research complied with the guidelines of the Canadian Council on Animal Care and the Animal Behavior Society. The experiments were conducted under McMaster University animal utilization protocol 18-01-02 and the University of Victoria animal utilization protocol Juanes-003.

## Results

### *Experiment 1: Physiological Response to Hypoxia and Air Exposure*

Increasing values of PC1 were linked with low $pH_i$ and glycogen content as well as high levels of lactate and free glucose across tissues, suggesting that high values of PC1 reflected recruitment of anaerobic metabolism and glucose mobilization throughout the body. Air-exposed fish had significantly elevated PC1 scores compared to normoxia-exposed fish (LMM, estimate [est.] ± SE = −4.00 ± 0.49, $Z = -8.12$, $P < 0.001$; fig. 1*d*, 1*e*) and hypoxia-exposed fish (LMM, est. ± SE = −2.60 ± 0.49, $Z = -5.28$, $P < 0.001$; fig. 1*d*, 1*e*). Hypoxia-exposed fish also had higher PC1 scores than normoxia-exposed fish (LMM, est. ± SE = −1.40 ± 0.49, $Z = -2.84$, $P = 0.012$; fig. 1*d*, 1*e*). PC2 was associated with increasing $pH_i$, and scores were higher in

air-exposed fish compared with normoxia-exposed fish (LMM, est. ± SE = −1.30 ± 0.33, $Z = -3.99$, $P < 0.001$; fig. 1*e*) but not hypoxia-exposed fish (LMM, est. ± SE = −0.49 ± 0.33, $Z = -1.51$, $P = 0.29$; fig 1*e*). PC2 values were higher in hypoxia-exposed fish than in normoxia-exposed fish (LMM, est. ± SE = −0.81 ± 0.33, $Z = -2.47$, $P = 0.036$; fig. 1*e*). There was no evidence that date of trial affected PC1 or PC2 response (LMM, Julian date, $P > 0.38$). Additionally, we examined the effect of treatment on individual physiological endpoints by using separate statistical tests, the results of which are summarized in tables 1 and A3 and are illustrated in figures A3–A5.

Hematocrit was higher in air-exposed fish (30.1%) than in normoxia-exposed fish (18.6%; LMM, est. ± SE = −11.51 ± 2.21, $Z = -5.20$, $P < 0.001$; fig. 2*a*) and hypoxia-exposed fish (22.1%; LMM, est. ± SE = −8.02 ± 2.25, $Z = -3.56$, $P = 0.001$; fig. 2*a*). This contributed to whole-blood Hb concentration being higher in air-exposed fish than in normoxia-exposed fish (LMM, est. ± SE = −1.19 ± 0.47, $Z = -2.52$, $P = 0.031$; fig. 2*b*), although not significantly higher than in hypoxia-exposed fish (LMM, est. ± SE = −0.65 ± 0.47, $Z = -1.37$, $P = 0.36$; fig. 2*b*). Additionally, mean cell Hb concentration did not differ between treatments (LMM, $P > 0.57$), suggesting that variation in blood Hb content was driven largely by changes in hematocrit and not the amount of Hb per cell volume.

Guarder males appeared to draw $O_2$ from their swim bladders during air exposure. Mass-standardized swim bladder $O_2$ content was lower in air-exposed fish (0.030 mL $O_2$ $g^{-1}$) than in normoxia-exposed fish (0.041 mL $O_2$ $g^{-1}$; LMM, est. ± SE = 1.41 ± 0.040, $Z = 3.49$, $P = 0.001$; fig. 2*c*) and hypoxia-exposed fish (0.037 mL $O_2$ $g^{-1}$; LMM, est. ± SE = 0.99 ± 0.040, $Z = 2.45$, $P = 0.037$; fig. 2*c*). Additionally, air-exposed fish had smaller swim bladder volumes than hypoxia-exposed fish (LMM, est. ± SE = 1.22 ± 0.47, $Z = 2.58$, $P = 0.027$; fig. 2*d*) but not normoxia-exposed fish (LMM, est. ± SE = 1.00 ± 0.47, $Z = 2.11$, $P = 0.087$; fig. 2*d*).

### *Experiment 2: Respiratory Response to Hypoxia*

Gill respiration changed with decreasing DO in two ways, both of which might have allowed guarder males to increase $O_2$ uptake during hypoxia. First, gill ventilation frequency increased as DO

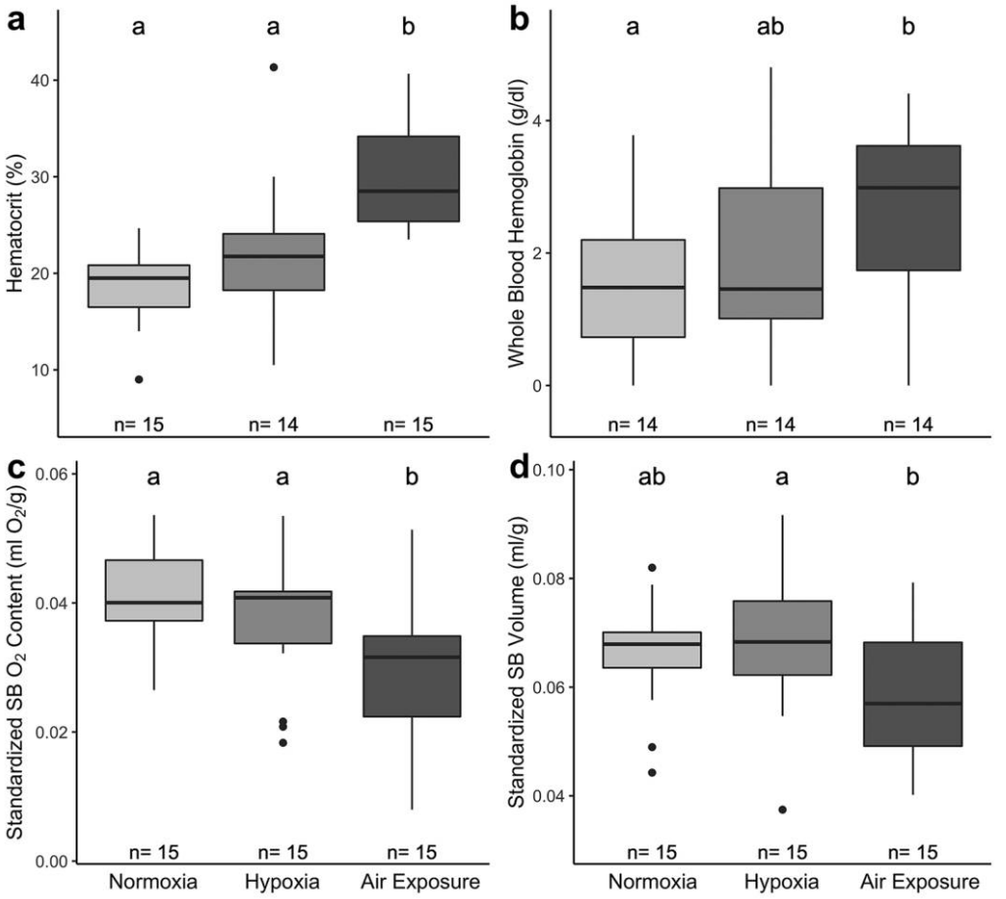


Figure 2. Effect of environmental treatment on hematocrit (*a*), whole-blood hemoglobin concentration (*b*), standardized swim bladder (SB) $O_2$ content (*c*), and standardized SB volume (*d*). Different letters above each boxplot denote significant differences at the level of $P < 0.05$ from post hoc Tukey tests.

dropped (LMM, $t = -9.03$, $P < 0.001$; fig. 3*c*). Second, fish performed ASR more frequently in hypoxia than normoxia trials (GLMM, $Z = 2.456$, $P = 0.014$). Although fish had access to a ramp that rose above the water in this experiment, they were never observed to exhibit voluntary air emersion (partial or full) during hypoxia.

## Discussion

Guarder males of the plainfin midshipman provide extended parental care in the intertidal zone where they are exposed to regular bouts of aquatic hypoxia and air exposure. We investigated the physiological consequences associated with this breeding strategy through controlled laboratory exposure of guarder males to aquatic hypoxia or air exposure, mimicking conditions in the wild. We found that plainfin midshipman display moderate physiological responses to bouts of hypoxia and air exposure. The physiological responses were captured by PC1 and PC2 (fig. 1*d*, 1*e*), driven by increased lactate and glucose levels and decreased glycogen levels across several tissues (table 1), thus suggesting some recruitment of anaerobic metabolism. Plainfin midshipman also seemed to rely on increases in blood $O_2$ carrying capacity (via increased whole-blood Hb concentration; fig. 2*b*), gill ventilation frequency (fig. 3), and ASR to cope with the simulated low-tide conditions. However, based on the modest magnitude of the changes in metabolite concentrations that we observed, we suggest that bouts of aquatic hypoxia and air exposure similar to those that plainfin midshipman fish regularly experience in the wild represent only moderate physiological challenges for these fish and that they are well suited to cope with the dynamic $O_2$ environment of the intertidal zone.

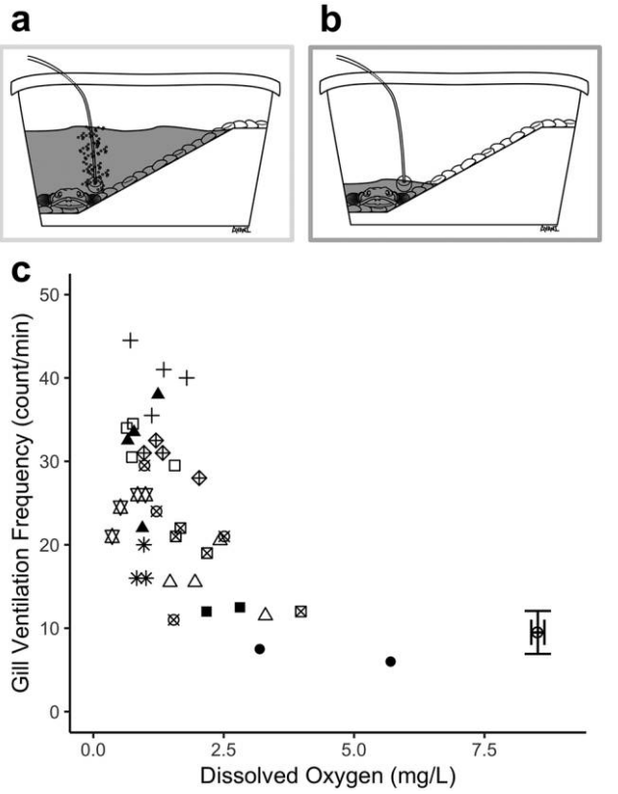


Figure 3. Illustrations of the normoxia (*a*) and hypoxia (*b*) treatments from experiment 2. *c*, Gill ventilation frequency measured at decreasing dissolved oxygen (DO) content across hypoxia and normoxia trials. The open circle represents an average value for all normoxia measurements ($n = 7$), with bidirectional error bars denoting standard deviations. Other symbols represent particular individuals measured repeatedly at different DO levels.

Recruitment of anaerobic metabolism can help fish maintain ATP homeostasis during aquatic hypoxia but can lead to lactate accumulation, glycogen depletion, and metabolic acidosis (Milligan and Wood 1986; Richards 2011). In our study, elevated muscle and plasma lactate content in hypoxia- and air-exposed fish (table 1) suggest that plainfin midshipman guarder males increase their reliance on anaerobic metabolism in low-$O_2$ conditions. The plainfin midshipman exposed to hypoxia and air exposure in our study displayed similarly modest increases in lactate concentration in their tissues compared to fish of the same species sampled in the field several hours after being isolated from the ocean by the receding tides (Bose et al. 2019). Another previous study has shown increased heart (but not muscle) lactate content during aquatic hypoxia in plainfin midshipman (Craig et al. 2014). Overall, recruitment of anaerobic metabolism appeared to be moderate in guarder males. We observed no evidence of metabolic acidosis (i.e., no clear drop in $pH_i$) in any of the examined tissues, and, consistent with previous studies on hypoxia-exposed plainfin midshipman guarder males, tissue glycogen levels were generally maintained, and the absolute magnitude of lactate increases in hypoxia- and air-exposed fish were low compared to what has been observed in other fishes exposed to similar conditions (Dunn and Hochachka 1986; Speers-Roesch et al. 2013; Craig et al. 2014; LaMoine et al. 2014). For example, plasma and white muscle lactate levels in excess of 6 mmol $L^{-1}$ and 11 $\mu$mol $g^{-1}$, respectively, have been reported in rainbow trout (*Oncorhynchus mykiss*) exposed to 3 h of acute aquatic hypoxia (DO = 1.7 mg $L^{-1}$; Dunn and Hochachka 1986). These values are much higher than those we observed in plainfin midshipman after 6 h of aquatic hypoxia (0.43 mmol $L^{-1}$ in plasma and 1.42 $\mu$mol $g^{-1}$ in muscle) or air exposure (1.4 mmol $L^{-1}$ in plasma and 1.64 $\mu$mol $g^{-1}$ in muscle; table 1). Plainfin midshipman also appear to recruit anaerobic metabolism to a lesser extent than other intertidal fishes with overlapping geographical ranges. For example, silverspotted sculpin (*Blepsias cirrhosus*), staghorn sculpin (*Leptocottus armatus*), and tidepool sculpin (*Oligocottus maculosus*) exposed to 6 h of relative aquatic hypoxia (30% of the average $P_{crit}$ for each species) all yielded lactate levels over 6 $\mu$mol $g^{-1}$ in the brain and 5 $\mu$mol $g^{-1}$ in white muscle (Speers-Roesch et al. 2013), far above hypoxia- and air-exposed plainfin midshipman fish (table 1). Relatively low levels of lactate and no development of metabolic acidosis despite a 6-h exposure to aquatic hypoxia or air suggest that plainfin midshipman might avoid substantial recruitment of anaerobic glycolysis during routine periods of low $O_2$ availability in the intertidal zone.

Alternatively, plainfin midshipman could rely heavily on anaerobic metabolism in a subset of tissues during hypoxia or

air exposure, if it is coupled to a concurrent increase in lactate disposal in other tissues, and thereby maintain low tissue lactate content despite elevated rates of anaerobic metabolism. Lactate disposal increases in fish during hypoxia and relies on either lactate oxidation (via entrance into the tricarboxylic acid cycle) or gluconeogenesis (Omlin and Weber 2010). Several studies on mammals and fish show that lactate is used preferentially over glucose as an oxidative fuel source in heart and brain tissue when lactate concentration is elevated (Drake et al. 1980; Lanctin et al. 1980; Bouzier-Sore et al. 2003, 2006), and high rates of lactate oxidation in these organs could potentially help maintain low lactate levels across the body as a whole (Milligan and Girard 1993; Omlin and Weber 2010; Omlin et al. 2014). Alternatively, plainfin midshipman guarder males could dispose of lactate via gluconeogenesis in the liver (Weber et al. 1986; Walsh 1989), as hypoxia can stimulate gluconeogenesis by increasing the activity of its requisite enzymes in some fishes (Wright et al. 1989; Gracey et al. 2001; Borowiec et al. 2015). Consistent with the idea that gluconeogenesis supported by lactate occurred during air exposure, glucose content in the liver was elevated by >300% in air-exposed fish relative to normoxia-exposed fish, and this occurred without depletion of tissue glycogen levels, so another source of increased glucose, such as gluconeogenesis, is likely. Increasing lactate disposal via lactate oxidation or gluconeogenesis in some tissues (heart and brain and/or liver, respectively) may help guarder males maintain ATP balance by using anaerobic metabolism in some others (e.g., muscle) while avoiding costs associated with lactate buildup and metabolic acidosis.

Plainfin midshipman might also limit the demand for anaerobic metabolism during aquatic hypoxia and/or air exposure through physiological strategies that help maintain aerobic metabolism. In our study, air exposure increased hematocrit (fig. 2*a*), consistent with previous work showing that guarder males increase hematocrit in response to acute hypoxia exposure (Craig et al. 2014). Air exposure also increased blood Hb concentration (fig. 2*b*), and hypoxia exposure increased gill ventilation frequency (fig. 3*c*) and the use of ASR. These responses are common in hypoxia-exposed fishes (Hughes 1973; Chapman and McKenzie 2009; Richards 2011) and serve to increase circulatory $O_2$ transport capacity (Nikinmaa and Soivio 1982; Claireaux et al. 1988) and/or brachial $O_2$ uptake (Shingles et al. 2005; Chapman and McKenzie 2009; Richards 2009). Plainfin midshipman might also extract limited amounts of $O_2$ through their skin and gills during air exposure (Martin 1993), though the extent to which their gills remain functional out of water is presently unknown. In non-amphibious fishes, gill filaments and lamellae generally collapse and coalesce out of water, drastically reducing the surface area available for gas exchange (Wright and Turko 2016). Additionally, we found reduced swim bladder $O_2$ content under hypoxia and air exposure (fig. 2*c*), which has also been demonstrated in plainfin midshipman following low-tide events in the field (Bose et al. 2019) and in the European eel (*Anguilla anguilla*; Berg and Steen 1965) and longjaw mudsucker (*Gillichthys mirabilis*; Todd and Ebeling 1966) in response to hypoxia. These fish might therefore draw $O_2$ from their swim bladders when they cannot extract sufficient $O_2$ through their gills or skin. However, in the current study, air-exposed fish lost $O_2$ from the swim bladder at an average rate of 0.086 mmol $O_2$ $kg^{-1}$ $h^{-1}$, which represents only ~4% of the resting metabolic rate of plainfin midshipman guarder males in normoxic conditions (~2.00 mmol $O_2$ $kg^{-1}$ $h^{-1}$; Craig et al. 2014). This suggests that $O_2$ from the swim bladders of air-exposed fish could not support the resting metabolism of guarder males on its own (though it could be important during periods of metabolic depression at low tide) and that other strategies for overcoming $O_2$ deprivation during air exposure play a more important role.

The third coping strategy used by many aquatic organisms facing low-$O_2$ conditions is metabolic depression (Boutilier 2001; Bickler and Buck 2007). Although metabolic rate was not measured in the present study, previous observations and studies suggest that plainfin midshipman reduce their metabolic rate during hypoxia (Arora 1948; Craig et al. 2014; LaMoine et al. 2014). Plainfin midshipman are generally sluggish and struggle little when found underneath nests at low tide (Arora 1948; N. Houpt, unpublished observation), suggesting that these fish are inactive in low-$O_2$ conditions. Additionally, plainfin midshipman exposed to hypoxia reduce their $O_2$ consumption ($M_{O_2}$) and nitrogenous waste excretion, suggesting lower protein turnover—a common mechanism of metabolic depression (Lewis et al. 2007; Craig et al. 2014; LaMoine et al. 2014). Similarly, hypoxia-exposed guarder males reduce the activity of gill sodium-potassium ATPase, which could facilitate metabolic depression by reducing the cost of compensatory ion pumping (Craig et al. 2014). Other marine fish, such as triplefin blenny (*Helcogramma medium*) and the intertidal epaulette sharks (*Hemiscyllium ocellatum*), are capable of depressing their metabolic rate when exposed to hypoxia (Innes and Wells 1985; Routley et al. 2002). While a comparison of rates of both aquatic and aerial $O_2$ consumption in plainfin midshipman in normoxia, hypoxia, and air exposure is still needed, current evidence suggests that $M_{O_2}$ depression is an important strategy used by these fish to maintain ATP homeostasis in hypoxia and air exposure without excessive reliance on anaerobic metabolism.

Among resident intertidal fishes, resilience to intertidal conditions is hypothesized to have arisen in multiple lineages in coevolution with parental guarding of beach-spawned eggs (Martin and Swiderski 2001; Martin et al. 2004). These adaptions allow species to tolerate an environment that benefits their offspring (Martin 2015). The plainfin midshipman presents a useful opportunity to investigate the possible coevolutionary relationship between physiological tolerance to intertidal conditions and intertidal egg guarding, as the toadfishes are the only family of subtidal residents that remain in the nest to guard eggs through low tides (Coleman 1999; Martin 2015). Among beach-spawning subtidal fishes, few remain guarding their eggs at low tide, and, accordingly, few exhibit physiological and behavioral strategies for withstanding low-tide conditions (Martin et al. 2004; Martin 2015). Plainfin midshipman guarder males are an exception to these tendencies (Coleman 1999; Martin et al. 2004; Martin 2015). Unlike other beach-spawning subtidal fishes, which typically abandon their eggs entirely after spawning, plainfin midshipman guard their eggs continuously for more than 60 d and endure

regular periods of hypoxia and air exposure (Coleman 1999; Martin et al. 2004; Cogliati et al. 2013; Martin 2015). Additionally, plainfin midshipman guarder males are similar to resident intertidal fishes in that they withstand long periods of air exposure (present study) and are tolerant of hypoxic conditions (Craig et al. 2014; LaMoine et al. 2014; Bose et al. 2019). However, the plainfin midshipman lacks the active behavioral emergence response to hypoxia that is common to other intertidal residents (Martin et al. 1996; Mandic et al. 2009; Richards 2011). The combination of responses that are characteristic to both intertidal and subtidal residents exhibited by the plainfin midshipman adds further evidence to the hypothesis that parental care of eggs might be the most important evolutionary driver of physiological tolerance to intertidal conditions (Martin et al. 2004).

Despite the dynamic nature of their intertidal nesting habitat, we show that plainfin midshipman guarder males suffer only modest physiological disturbance in response to the short-term environmental fluctuations across the tidal cycle. However, guarder males still suffer severe energetic costs while rearing their young (Bose et al. 2014, 2019). During their parental care period, plainfin midshipman guarder males suffer reduced body condition and endogenous fuel reserves (e.g., reductions in liver glycogen and lipid contents and muscle protein content; Bose et al. 2014, 2015). This could be the result of the intensive (and potentially metabolically costly) parental care provided by these fish, as guarder males at high tide regularly inspect and clean their young, constantly maintain their nesting cavity, and continually defend their nests from takeovers from rival males or other invaders (such as red rock crabs, *Cancer productus*; Arora 1948; N. Brown, N. Houpt, N. Yee, J. Curtis, B. Bolker, F. Juanes, and S. Balshine, unpublished manuscript). Plainfin midshipman and other toadfish are unique among subtidal-resident intertidally breeding fish in providing extended posthatching parental care (Coleman 1999), and their physiological resilience in the face of harsh intertidal conditions might allow them to do so for such an unusually prolonged duration.

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## APPENDIX

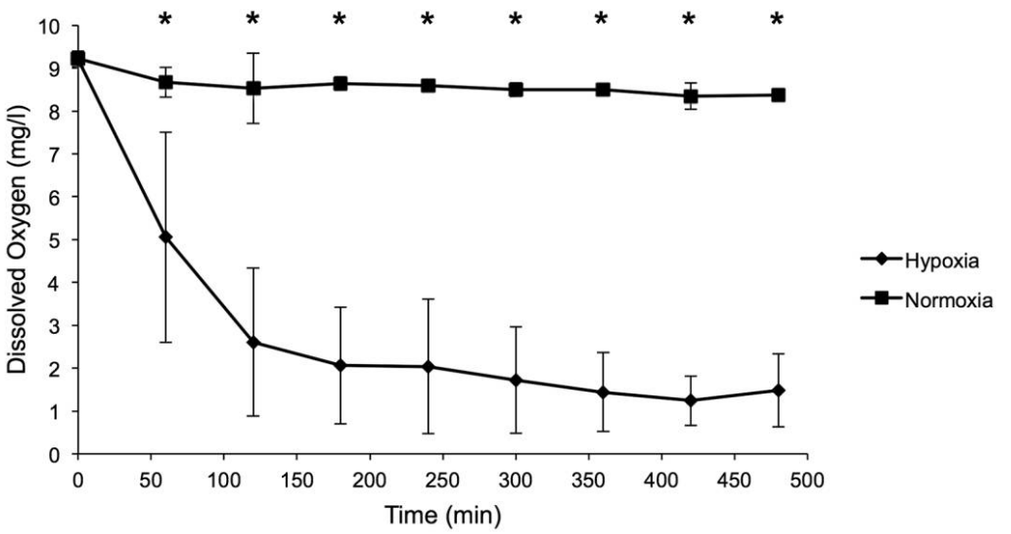


Figure A1. Average dissolved oxygen (DO) content measured hourly during normoxia and hypoxia trials in experiment 2. Error bars denote ± SD. Asterisks denote significant differences in DO between treatments at the level of $P < 0.05$ from two-sample *t*-tests.

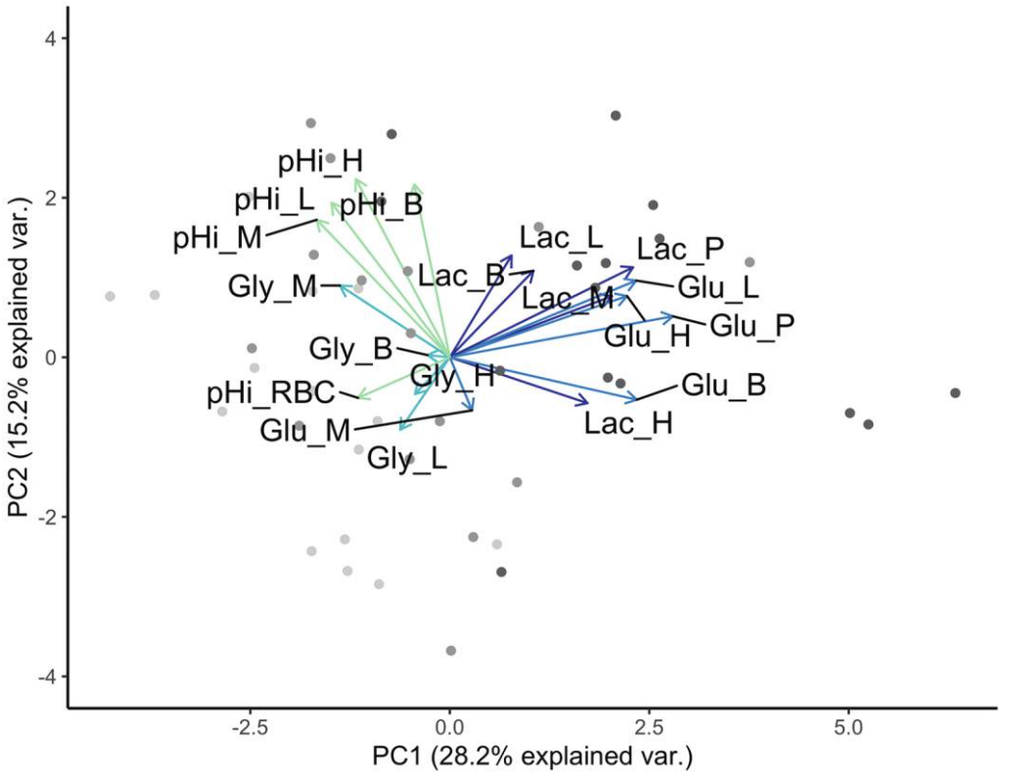


Figure A2. Graphical representations of principal component 1 (PC1) and 2 (PC2) loadings. Colors represent metabolite type (lactate [Lac] = purple; intracellular pH [$pH_i$] = green; glucose [Glu] = dark blue; glycogen [Gly] = light blue), and tissue types are abbreviated as follows: P = plasma; M = muscle; H = heart; L = liver; B = brain; RBC = red blood cell.

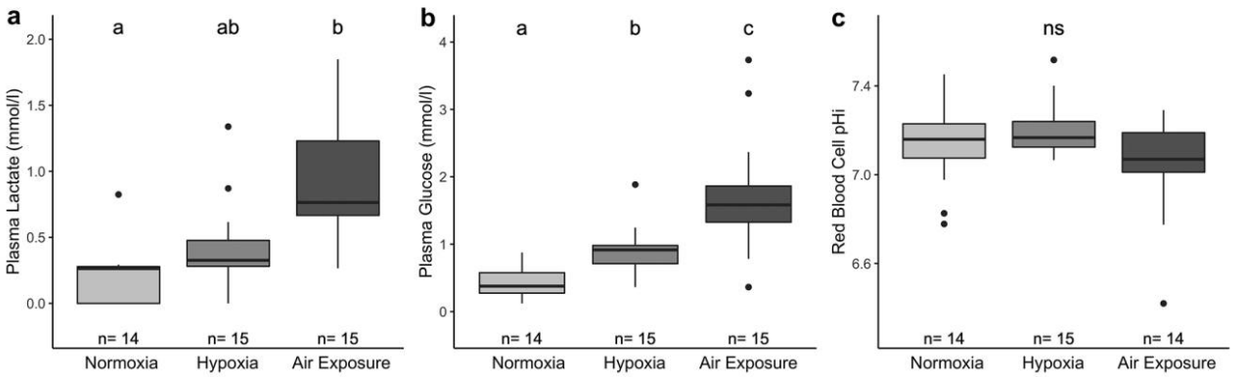


Figure A3. Plasma and red blood cell (RBC) metabolite levels of fish following a 6-h normoxia, hypoxia, or air exposure trial. Figure depicts lactate (*a*) and glucose content (*b*) in the plasma of exposed fish. *c*, RBC intracellular pH. Different letters above each boxplot denote significant differences at the level of $P < 0.05$ from post hoc Tukey tests.

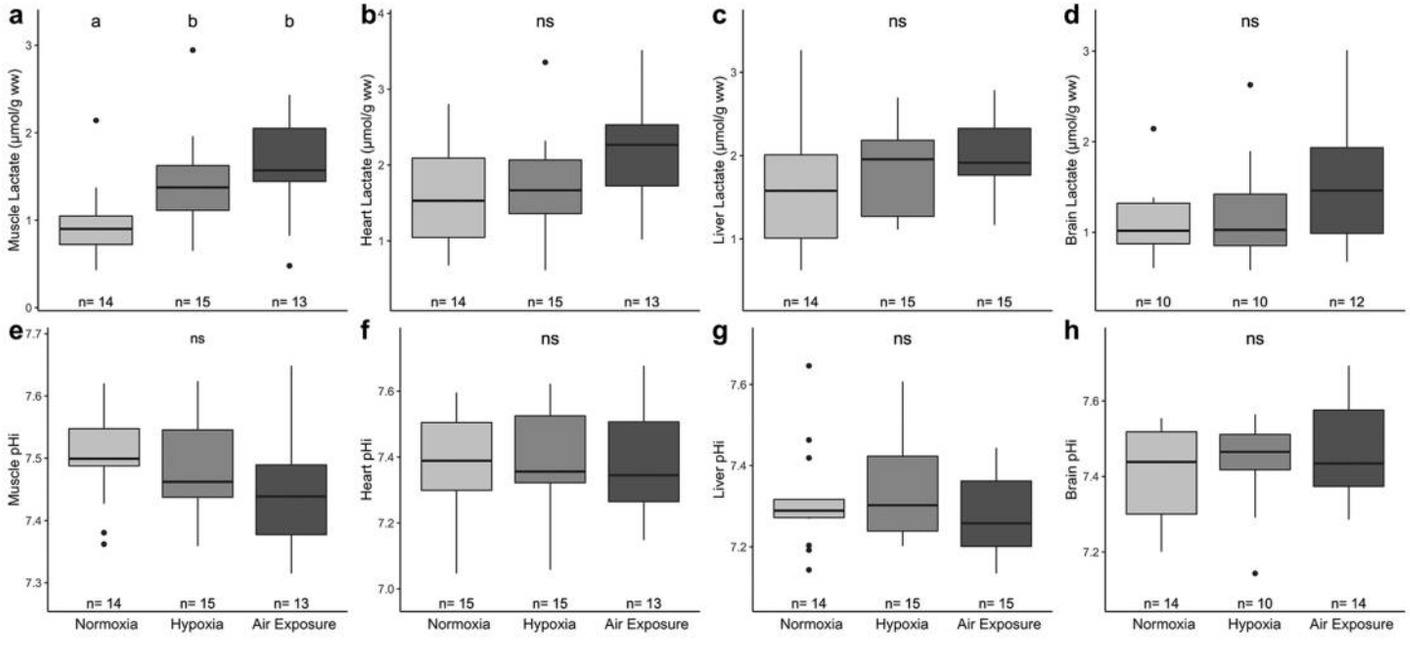


Figure A4. Lactate and intracellular pH ($pH_i$) response of fish to normoxia, hypoxia, or air exposure in muscle, heart, liver, and brain tissue. Figure depicts lactate content in the muscle (*a*), heart (*b*), liver (*c*), and brain (*d*) and $pH_i$ across three exposures in the muscle (*e*), heart (*f*), liver (*g*), and brain (*h*). Different letters above each boxplot denote significant differences at the level of $P < 0.05$ from post hoc Tukey tests.

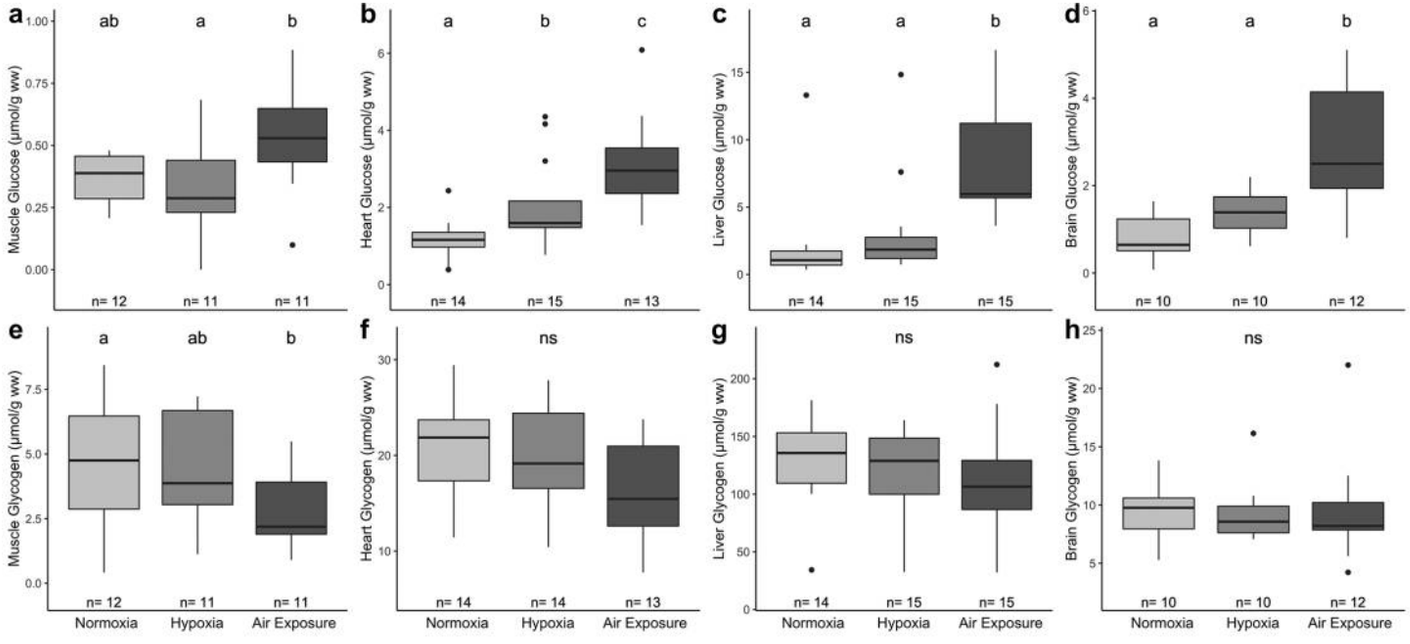


Figure A5. Energetic response in muscle, heart, liver, and brain tissue of fish to normoxia, hypoxia, and air exposure. Figure depicts glucose content in fish exposed to simulated tide conditions in muscle (*a*), heart (*b*), liver (*c*), and brain tissue (*d*) and glycogen content in the muscle (*e*), heart (*f*), liver (*g*), and brain (*h*). Different letters above each boxplot denote significant differences at the level of $P < 0.05$ from post hoc Tukey tests.

Table A1: Guarder male measurements and water quality summary information for experiment 1

| Treatment | Average fish mass (g) | Standard length (cm) | Fulton's condition factor (*K*) | Temperature (°C) | | Dissolved oxygen (mg $L^{-1}$) | | Ammonia (ppm) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Initial | Final | Initial | Final | Initial | Final |
| Normoxia | 110.5 ± 23.3 (77.8–147.6) | 20.4 ± 1.7 (17.5–22.9) | 1.30 ± .09 (1.15–1.45) | 12.93 ± .31 (12.5–13.8) | 13.01 ± .43 (12.4–13.9) | 9.16 ± .17 (8.84–9.41) | 9.09 ± .13 (9.01–9.23) | 0 ± 0 (0–0) | .03 ± .09 (0–.25) |
| Hypoxia | 111.7 ± 23.4 (76.1–154.5) | 20.4 ± 1.7 (17.9–23.4) | 1.30 ± .10 (1.12–1.44) | 12.93 ± .30 (12.5–13.5) | 13.96 ± .93 (12.9–16.3) | 9.13 ± .16 (8.83–9.35) | 4.23 ± 2.12 (8.21–2.09) | 0 ± 0 (0–0) | .08 ± .12 (0–.25) |
| Air exposure | 112.1 ± 24.7 (76.6–155.3) | 20.5 ± 1.5 (18.4–23.2) | 1.29 ± .14 (1.05–1.51) | 12.91 ± .30 (12.5–13.4) | 16.68 ± 1.32 (14.3–18.8) | 9.15 ± .15 (8.91–9.43) | … | 0 ± 0 (0–0) | … |

Note. Mean ± SD shown for each variable, with range shown in parentheses.

Table A2: Guarder male measurements for experiment 2

| Measurement term | Measurement amount |
|---|---|
| Average fish mass (g) | 92.2 ± 28.7 (50.86–142.8) |
| Standard length (cm) | 19.4 ± 2.0 (16.8–22.8) |
| Fulton's condition factor (*K*) | 1.24 ± .19 (.98–1.73) |

Note. Mean ± SD shown for each variable, with range shown in parentheses.

Table A3: Statistical output of Tukey tests for metabolic endpoints from experiment 1

| Metabolites | Normoxia-hypoxia | Normoxia–air exposure | Hypoxia–air exposure |
|---|---|---|---|
| Lactate: | | | |
| Plasma | −.32 ± .25, $Z = -1.26$, $P = .42$ | −1.25 ± .25, $Z = -4.94$, $P < .001$* | −.93 ± .25, $Z = -3.74$, $P < .001$* |
| Muscle | −.45 ± .17, $Z = -2.44$, $P = .040$* | −.67 ± .19, $Z = -3.46$, $P = .002$* | −.22 ± .19, $Z = -1.13$, $P = .49$ |
| Heart | −.074 ± .23, $Z = -.32$, $P = .94$ | −.59 ± .24, $Z = -2.49$, $P = .034$* | −.52 ± .23, $Z = -2.22$, $P = .068$ |
| Liver | −.25 ± .20, $Z = -1.24$, $P = .43$ | −.42 ± .20, $Z = -2.11$, $P = .087$ | −.18 ± .20, $Z = -.89$, $P = .65$ |
| Brain | −.38 ± .28, $Z = -1.36$, $P = .36$ | −.45 ± .28, $Z = -1.63$, $P = .24$ | −.07 ± .29, $Z = -.25$, $P = .97$ |
| $pH_i$: | | | |
| Muscle | .0054 ± .018, $Z = .30$, $P = .95$ | .040 ± .019, $Z = 2.11$, $P = .089$ | .034 ± .018, $Z = 1.86$, $P = .15$ |
| Heart | −.0063 ± .037, $Z = -.17$, $P = .98$ | .0016 ± .039, $Z = .43$, $P = .90$ | .023 ± .039, $Z = .59$, $P = .82$ |
| Liver | −.0028 ± .0041, $Z = -.68$, $P = .77$ | .0048 ± .0041, $Z = 1.19$, $P = .46$ | .0076 ± .0040, $Z = 1.92$, $P = .13$ |
| Brain | −.024 ± .040, $Z = -.60$, $P = .82$ | −.053 ± .036, $Z = -1.48$, $P = .30$ | −.030 ± .040, $Z = -.74$, $P = .74$ |
| RBC | .16 ± .070, $Z = 2.32$, $P = .054$ | .11 ± .071, $Z = 1.49$, $P = .29$ | −.056 ± .070, $Z = -.80$, $P = .71$ |
| Glucose: | | | |
| Plasma | −.53 ± .13, $Z = -3.98$, $P < .001$* | −1.03 ± .13, $Z = -7.70$, $P < .001$* | −.50 ± .13, $Z = -3.79$, $P < .001$* |
| Muscle | .032 ± .076, $Z = .43$, $P = .90$ | −.17 ± .076, $Z = -2.26$, $P = .062$ | −.20 ± .77, $Z = -2.63$, $P = .023$* |
| Heart | −.33 ± .10, $Z = -3.27$, $P = .003$* | −.70 ± .10, $Z = -6.67$, $P < .001$* | −.37 ± .10, $Z = -3.61$, $P < .001$* |
| Liver | −1.07 ± 1.32, $Z = -.81$, $P = .70$ | −6.08 ± 1.32, $Z = -4.62$, $P < .001$* | −5.01 ± 1.29, $Z = -3.88$, $P < .001$* |
| Brain | −1.52 ± .41, $Z = -3.71$, $P < .001$* | −2.12 ± .41, $Z = -5.16$, $P < .001$* | −.60 ± .043, $Z = -1.39$, $P = .35$ |
| Glycogen: | | | |
| Muscle | .20 ± .81, $Z = .25$, $P = .97$ | 1.98 ± .81, $Z = 2.44$, $P = .039$* | 1.78 ± .83, $Z = 2.14$, $P = .082$ |
| Heart | 1.20 ± 2.09, $Z = .58$, $P = .83$ | 4.71 ± 2.13, $Z = 2.21$, $P = .070$ | 3.50 ± 2.13, $Z = 1.64$, $P = .23$ |
| Liver | 8.16 ± 14.9, $Z = .55$, $P = .85$ | 16.6 ± 14.9, $Z = 1.11$, $P = .51$ | 8.41 ± 14.6, $Z = .57$, $P = .83$ |
| Brain | −.28 ± 1.45, $Z = -.19$, $P = .98$ | −.16 ± 1.45, $Z = -.11$, $P = .99$ | .12 ± 1.51, $Z = .08$, $P = 1.00$ |

Note. Tukey tests were run based on linear mixed effects models created for each endpoint. Estimate ± SE is given first, followed by *Z* and *P* values. $pH_i$ = intracellular pH; RBC = red blood cell.

*Significant differences at the level of $P < 0.05$.

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