Divergent Hypoxia Tolerance in Adult Males and Females of the Plainfin Midshipman (*Porichthys notatus*)

Author(s): Christophe M. R. LeMoine, Carol Bucking, Paul M. Craig, and Patrick J. Walsh

Source: *Physiological and Biochemical Zoology*, Vol. 87, No. 2 (March/April), pp. 325-333
Published by: The University of Chicago Press
Stable URL: http://www.jstor.org/stable/10.1086/674565

Divergent Hypoxia Tolerance in Adult Males and Females of the Plainfin Midshipman (*Porichthys notatus*)

Christophe M. R. LeMoine*
Carol Bucking
Paul M. Craig
Patrick J. Walsh
Department of Biology and Center for Advanced Research in Environmental Genomics, University of Ottawa, 30 Marie Curie Private, Ottawa, Ontario K1N 6N5, Canada, and Bamfield Marine Sciences Centre, 100 Pachena Drive, Bamfield, British Columbia V0R 1B0, Canada

Accepted 9/13/2013; Electronically Published 2/5/2014

ABSTRACT

In the summer, the plainfin midshipman (*Poricthhys notatus*) migrates to reproduce in the nearshore environment, where oxygen levels are influenced by the tidal cycles. Parental males establish nests under rocks in the intertidal zone, where they reside until the eggs they guard are fully developed. In contrast, females and sneaker males leave the nests shortly after spawning. We examined the physiological resistance and metabolic response of parental male and female adult midshipman to hypoxia to test whether they exhibited sex-specific differences reflecting their reproductive strategies. Further, we assessed whether metabolic enzymes and metabolites were differentially enriched in tissues of parental males and females to explain the differences observed in their hypoxia tolerance. While parental males and females exhibited similar depression of their oxygen consumption in response to graded hypoxia, parental males could withstand significantly longer exposures to severe hypoxic stress. At the biochemical level, parental males showed higher hepatic glycogen reserves and higher glycolytic enzyme capacities in gills and skeletal muscles than females. Although some of these enzymatic variations could be explained by differences in body size, we also observed a significant effect of sex on some of these factors. These results suggest that parental male midshipman may benefit from sexual dimorphism at the whole-organismal (larger body size) and biochemical (enzyme activities) levels, conferring on them a higher glycolytic potential to sustain the extensive hypoxia bouts they experience in nature.

* Corresponding author; e-mail: clemoine@uottawa.ca.

Physiological and Biochemical Zoology 87(2):325–333. 2014. 1522-2152/2014/8702-2134$15.00. DOI: 10.1086/674565

Introduction

The plainfin midshipman (*Poricthhys notatus*, Girard), family Batrachoididae, is a demersal marine species found along the eastern Pacific coast of North America (Arora 1948; Walker and Rosenblatt 1988). During their reproductive season, midshipman migrate from their open sea environment to intertidal zones where they court and spawn (Feder et al. 1974). Adult *P. notatus* can be categorized in three reproductive morphs based on their morphometry and their distinct behavioral and reproductive strategies (Brantley and Bass 1994). Parental males (type I) are the largest of the three morphs (51.6–218.1 g; this study) and are usually the first to colonize the intertidal zone (Brantley and Bass 1994). Once there, they select a rocky shelter where they establish and defend their nest. At night, parental males attract females (38.1–93.9 g; this study) by producing advertisement calls using a specialized sonic muscle surrounding their swim bladder (Bass et al. 1999). Type II, or sneaker, males bypass nest building and female courting by temporarily sneaking into established nests and stealing fertilization from parental males (Brantley and Bass 1994). Shortly after spawning, the females and sneaker males leave the breeding grounds to return to their offshore environment. Parental males, on the other hand, remain in the nest to provide parental care for the young until they develop into free-swimming larvae, approximately 30–40 d postfertilization (Arora 1948). Notably, multiple clutches can keep type I males in the intertidal zone for several summer months.

The nearshore marine environment is highly variable and fluctuates with the ebb and flow of tides (Truchot and Duhamel-Jouve 1980; Morris and Taylor 1983). At high tide, oxygen saturation is relatively close to that of open water, providing a stable normoxic environment. However, when the tide recedes, the tide pools left behind can experience a dramatic reduction in oxygen availability lasting several hours (Truchot and Duhamel-Jouve 1980). While some fish escape these variations by leaving with the tide, the reproductive cycle of the midshipman subjects them to these extreme conditions, particularly the parental males that remain in their nest (i.e., small pools under rocks) throughout the entire breeding season.

Resident organisms of the intertidal zone have developed a variety of adaptive strategies to survive daily environmental fluctuations. For example, several fish species, including *P. notatus*, use surface or aerial respiration to cope with severely hypoxic water (Davenport and Woolmington 1981; Martin

1993, 1996; Mandic et al. 2009). However, this strategy may prove dangerous for species that experience high rates of bird predation such as the midshipman (see DeMartini 1988). Other hypoxia-tolerant species cope with low O_2 availability by severely reducing their metabolic rate and by switching metabolic pathways to produce energy (Brauner et al. 1995; Muusze et al. 1998). Indeed, under aerobic conditions, most ATP is produced via oxidative pathways, but when oxygen becomes limiting, cells and tissues increase their reliance on less efficient anaerobic pathways for energy production. Thus, several hypoxia-tolerant species demonstrate a high glycolytic capacity that allows them to rely primarily on anaerobic ATP production in hypoxic conditions (e.g., Almeida-Val et al. 2000; Davies and Moyes 2007).

Previous work showed that nesting parental males experience intertidal hypoxia (Craig et al. 2014), which in view of their reproductive strategies suggests that, unlike females, they have to endure extended periods of hypoxia. Further, as these animals are hypoxia tolerant and rely more on anaerobic processes during hypoxic stress (as indicated by blood lactate levels; Craig et al. 2014), we hypothesized that type I males would be more resistant to environmental hypoxia than their female counterparts. We predicted that parental males would present metabolic adaptations allowing them to better withstand hypoxic bouts than female *P. notatus* and tested our prediction by subjecting parental males and females to severe hypoxia and graded hypoxia. Under these conditions, we examined a variety of metabolic indicators in these animals. Specifically, we examined their rates of oxygen consumption to assess the respiratory adjustments of the animals under hypoxia, predicting that *P. notatus* would exhibit a sustained and severe depression of their oxygen consumption rate in hypoxic conditions. As another indicator of metabolic status (i.e., amino acid catabolism), we assessed the nitrogen waste excretion rate of these animals during a graded hypoxia, predicting a reduction of these rates coincident with O_2 consumption rate depression. At the tissue level, we measured the activity of an important energy consumer, the sodium potassium ATPase (NKA; Rolfe and Brown 1997), predicting that under hypoxia this enzyme would be downregulated. Finally, we evaluated the levels of a marker enzyme of mitochondrial content, citrate synthase; two enzymes of anaerobic metabolism, pyruvate kinase and lactate dehydrogenase (Llorente et al. 1970; Jungmann et al. 1998; Muñoz and Ponce 2003); and the levels of glycolytic metabolites (lactate, glucose, and glycogen) to assess whether a higher potential for anaerobic metabolism could explain sex-specific differences in hypoxia tolerance. Our results suggest that the parental male midshipman have a biochemical advantage over females that may allow them to cope with the hypoxic environment they encounter during the breeding season.

Material and Methods

Animals

Plainfin midshipman were collected on nest sites in June 2011 and June 2012 from Nanoose Bay (49°15′N, 124°10′W) on Vancouver Island, British Columbia, Canada, under a collection permit from the Department of Fisheries and Oceans, Canada. We kept our sampling to the minimum to ensure that a majority of nests were left undisturbed or with at least one parental male guarding eggs. The fish were then transported in aerated seawater to the Bamfield Marine Science Centre, Bamfield, British Columbia, Canada. Upon arrival, the animals were weighed and allowed to acclimate for 2–4 wk in flow-through systems of aerated seawater tanks kept at approximately 11°C and not fed for the duration of the experiments, mimicking natural conditions (Coleman 1999). All procedures were approved by the University of Ottawa and Bamfield Marine Sciences Centre Animal Care Committees (protocol BL-255).

Severe Hypoxia Challenge

Fish were placed in 1- or 3-L mason jars, depending on their size, to minimize water volume and restrict their movements while not causing undue distress. The jars were equipped with individual air lines and the fish allowed to acclimate for 30 min. Temperature was maintained by placing the jars in a water bath (11°C). Once settled, the fish were subjected to a severe hypoxia challenge, where the water in the jars was replaced with hypoxic water and the jars were sealed. Hypoxic water was obtained from a separate tank of seawater that was bubbled with nitrogen gas throughout the duration of the experiment. Every hour, the water was replaced with fresh hypoxic water, and the oxygen partial pressure in the jars was monitored with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) and was determined to be 5%–8% of air saturation throughout the experiment. When changing the water, the responsiveness of the fish was assessed by a tail pinch. Failure to respond to the pinch indicated the endpoint of the experiment for that fish. The animals were then allowed to recover in oxygenated water.

Gradual Hypoxia Exposure

Fish ($n = 36$) were held in jars as described above, loosely closed with rubber stoppers with both an air line and a needle, allowing for sampling of the water with minimal disturbance of the fish, and allowed to acclimate in normoxia for a 4-h period. Water samples were taken at 2-h intervals during this acclimation to evaluate the rates of nitrogenous waste excretion. At the end of the acclimation period, the water was replaced, the air supply shut down, and the jar sealed, allowing the fish to gradually become hypoxic through their own oxygen consumption. During this hypoxic treatment, water samples were obtained every hour to measure nitrogenous waste excretion rates. As described above, the oxygen tension in the jars was measured every 30 min to compute individual fish oxygen consumption rates. At the end of this period, the water was replaced, and the jars were refitted with air supply and the fish allowed to recover for 4 h, during which nitrogenous waste excretion rates were evaluated every 2 h. Water ammonia and urea concentrations were assessed following established pro-

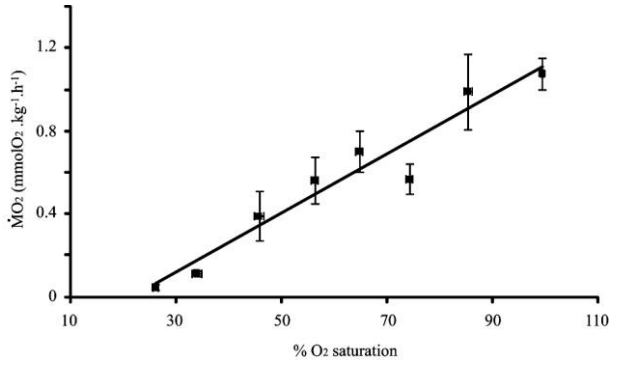

Figure 1. Oxygen consumption depression in midshipman exposed to gradual hypoxia. Oxygen saturation values for both sexes were binned and averaged every 10% to provide an average oxygen consumption for this percent O_2 saturation. The line represents a significant linear relationship between mass-specific oxygen consumption and O_2 concentration ($y = 0.01x - 0.30$, $r^2 = 0.94$).

tocols (Rahmatullah and Boyde 1980; Ivancic and Degobbis 1984).

Tissue Collection and Analysis

Tissues were collected from normoxic fish from the holding tanks and from animals sampled at the end of a 3-h gradual hypoxia exposure (see above). Fish were lethally anesthetized with an overdose of tricaine methanesulfonate (1 g/L). The animals were immediately weighed, and their gills, intestine, liver, brain, and mixed skeletal muscle were excised and flash frozen using freeze clamps in liquid nitrogen and stored at −80°C until further analysis. In addition, the weights of the whole liver (prior to freezing) were recorded for most animals. Inspection of the sonic muscle surrounding the swim bladder in males (highly differentiated in type I males) allowed identification of type I and type II males. As we were not able to collect a sufficient number of type II males for adequate statistical power, they were excluded from further analysis. Parental males collected over the two field seasons were bigger than females (112.8 ± 9.1 g vs. 61.2 ± 3.9 g) and had a larger liver than females (2.8 ± 0.3 g vs. 1.1 ± 0.1 g).

Tissues were powdered under liquid nitrogen prior to enzymatic and metabolite analyses. For metabolites, tissues were homogenized in 8% perchloric acid and neutralized with saturated KOH (lactate) or K_2CO_3 (glucose-glycogen) and assayed following standard procedures (Gawehn and Bergmeyer 1974; Passonneau and Lauderdale 1974). Metabolic enzyme analyses for citrate synthase (CS; EC 2.3.3.1), lactate dehydrogenase (LDH; EC 1.1.1.27), and pyruvate kinase (PK; EC 2.7.1.40) were performed on tissues homogenized in buffer (50 mM Hepes, 1 mM EDTA 0.1% Triton X, pH 7.0) and assayed as previously described (McClelland et al. 2005; LeMoine et al. 2008). The activity of Na^+/K^+-ATPase (NKA; EC 3.6.3.9) was determined in samples homogenized in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) containing 0.3% Na deoxycholic acid and assayed as previously described (McCormick 1993).

Statistical Analyses

Data are expressed as means ± SEM and were statistically analyzed using SigmaStat v 3.5 (Systat, San Jose, CA). Statistical significance ($P < 0.05$) was established using *t*-tests (for pairwise analysis) and two-way ANOVA (metabolites and enzymes data) or two-way repeated-measures ANOVA (nitrogenous waste data) followed by Holm-Sidak post hoc test when appropriate. The linear relationships between mass-specific oxygen consumption rate and oxygen tension and between enzyme activities and animal weights were analyzed using a general linear regression model followed by a *t*-test analysis of the residuals to test the influence of sex on these factors. In an attempt to assess P_{crit}, the oxygen tension at which an animal becomes an oxyconformer, we averaged percent oxygen tension and associated mass-specific oxygen consumption rates to obtain an average $\dot{M}O_2$ at 10% increments of oxygen saturation. As P_{crit} could not be obtained, the relationship between $\dot{M}O_2$ and O_2 saturation was subsequently analyzed using a general linear regression model.

Results

Hypoxia Challenges

We exposed type I parental males and females to a severe hypoxia challenge (<8% of air O_2 saturation at 11°C) to test their physiological resistance to this stressor. Females (73.7 ± 4.9 g, $n = 4$) were responsive to a tail pinch up to 5 h under these conditions. In contrast, parental males (98.4 ± 7.3 g, $n = 4$)

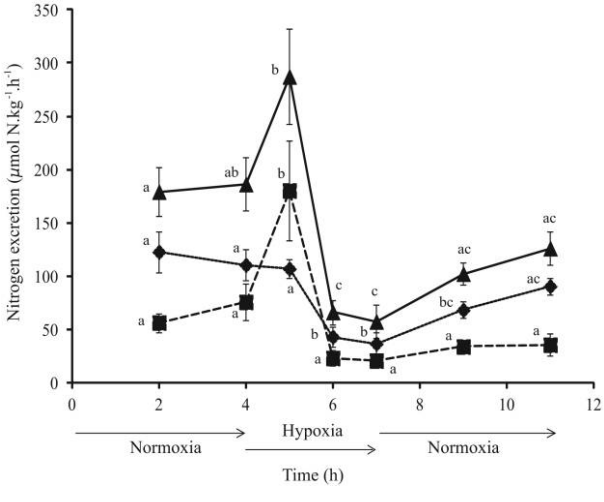

Figure 2. Nitrogenous waste excretion during hypoxia. Nitrogenous waste (triangles), ammonia (diamonds), and urea (squares) excretion rates were measured during normoxia, gradual hypoxia, and recovery in adult midshipman. Data are represented as means ± SEM. Different letters indicate statistically significant differences between time points ($n = 14$).

Table 1: Tissue glucose, glycogen, and lactate concentration during hypoxia in plainfin midshipman

	Males		Females	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Liver (μmol/g tissue):				
Glucose	8.89 ± 2.50^{A}	26.23 ± 7.25^{A,*}	3.36 ± .41^{B}	11.50 ± 2.17^{B,*}
Glycogen	540.52 ± 58.10^{A}	466.48 ± 77.48^{A}	139.36 ± 12.45^{B}	84.00 ± 25.81^{B}
Liver (μmol/whole liver):				
Glucose	44.78 ± 15.67^{A}	84.87 ± 15.67^{A,*}	3.68 ± .86^{B}	13.47 ± 2.07^{B,*}
Glycogen	2,410.04 ± 371.17^{A}	1,730.35 ± 476.17^{A}	173.91 ± 45.27^{B}	108.93 ± 41.22^{B}
Muscle (μmol/g tissue):				
Glucose	.83 ± .23	1.51 ± .29		
Glycogen	15.78 ± 2.55	15.25 ± 4.10		
Lactate	.92 ± .20	.10 ± .34		

Note. Metabolite levels were measured in tissues of parental (type I) male and female midshipman held under normoxia and after 3 h of gradual hypoxia exposure (see Material and Methods). Data are represented as means ± SEM. Different superscript letters indicate statistically significant differences between sexes, while an asterisk denotes a significant effect of hypoxia (n = 4–6).

were responsive for almost 10 h, twice as long as their female counterparts.

Given these differences in hypoxia tolerance, we investigated the metabolic response of these fish to a graded hypoxia challenge. The fish were allowed to gradually deplete the oxygen content in their holding water as we monitored their rates of oxygen consumption and nitrogen excretion. Overall, gradual hypoxia caused a decrease in mass-specific oxygen consumption rates (O_2) regardless of the sex of the animal as revealed by residual analysis (fig. 1; data not shown). By the end of the 3-h exposure, larger fish (155.72 ± 8.04 g) consumed enough oxygen to reduce O_2 saturation down to 26% of normoxic value (fig. 1), and on average their oxygen consumption rate was depressed down to 6% of their normoxic value (n = 8, 2 females and 6 males). We then looked at the oxygen tension at which O_2 consumption falls (P_{crit}) in males and females. In both sexes, the mass-specific oxygen consumption rate decreased linearly with oxygen tension (fig. 1), suggesting that *Poricthhys notatus* are oxyconformers over a wide range of oxygen tension.

In addition, we also measured the rates of nitrogen waste excretion in response to hypoxia. The patterns of ammonia, urea, and nitrogenous waste excretion rates were not sex specific; thus, we have reported the combined data for both sexes. After 2 h of hypoxia, ammonia excretion rate was reduced by 70% compared to normoxic values (fig. 2); it remained low for the remainder of the hypoxia exposure and slowly recovered to reach control values after 4 h of recovery (fig. 2). Urea excretion rate showed a transient increase at the onset of hypoxia but remained relatively constant and similar to normoxic levels throughout the rest of the hypoxia exposure and recovery period (fig. 2). Thus, largely due to changes in ammonia excretion rates, there was an overall decrease in total nitrogenous waste excretion rates 2 h after the initiation of hypoxia, which slowly returned toward control levels during the recovery period (fig. 2).

Biochemical Analyses

Considering the profound impact of gradual hypoxia on patterns of oxygen consumption rates and nitrogen excretion rates, we investigated whether metabolic biochemical parameters were also affected by a reduction in water oxygen content. In the liver, there was a threefold increase in glucose concentration in both males and females following hypoxia (table 1). In contrast, glycogen levels did not change in hypoxic livers of both sexes (table 1). When comparing males and females, hepatic glycogen reserves were approximately threefold higher in males than in females when estimated per gram of tissue (table 1). These differences were even more pronounced when related to the whole liver. In fact, total hepatic glucose and glycogen contents were 10-fold higher in males than in females (table 1) and sevenfold higher in males when reported per body mass (data not shown). In contrast, there were no apparent changes in glucose, glycogen, or lactate levels in skeletal muscle of parental males following hypoxia (table 1).

We also assessed the enzymatic activity of the NKA, an important consumer of cellular ATP, in several tissues (fig. 3). In females, there was no effect of hypoxia on NKA activity per milligram protein regardless of the tissue examined (fig. 3*A*). In contrast, while NKA activity did not change in brain or intestine of the male *P. notatus*, their gills exhibited a twofold increase in activity per protein content during hypoxia (fig. 3*B*). In addition, we explored whether enzymes associated with aerobic metabolism (CS) and glycolysis (LDH, PK) were altered by hypoxia in liver, gill, and muscle tissues (table 2). None of the enzymes examined were affected by hypoxic treatments in either males or females. In the liver, both LDH and PK had lower activities per gram of tissue in males than in females, but these differences disappeared when corrected for the total liver weight (table 2; data not shown). In contrast, branchial PK showed higher mass-specific activity in males than in females. Similarly, in skeletal muscle, males exhibited higher LDH enzyme activities (table 2). To further investigate these differences

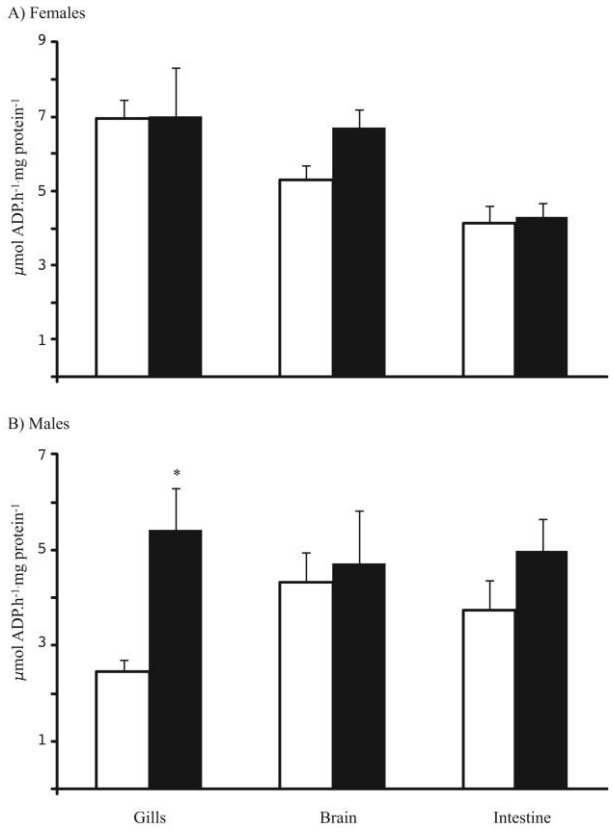

Figure 3. Na^+/K^+-ATPase activity in hypoxia. Na^+/K^+-ATPase was evaluated in gills, brain, and intestine from females (*A*) and parental males (*B*) exposed to normoxia (white bars) and hypoxia (black bars). Data are represented as means ± SEM. An asterisk denotes statistically significant differences between treatments (n = 4–6).

in gill PK and muscle LDH activity, we first looked at the effect of body size on mass-specific enzymatic capacity (fig. 4). In gills, there was a positive allometric relationship between PK activity and body size (r^2 = 0.22), while no such relationships were found for either CS or LDH (fig. 4; data not shown). Similarly in muscle, body weight was a good predictor of LDH activity (r^2 = 0.40) but not for PK or CS (fig. 4; data not shown). Using the residuals generated by these linear regressions, we evaluated whether sex had an influence on tissue-specific enzymatic activities when accounting for differences in body size. This analysis revealed that differences in LDH activities were not significantly influenced by the sex of the animal, while residual PK activity in gills was significantly elevated in males (0.026 ± 0.016, n = 19) compared to females (−0.063 ± 0.028, n = 8).

Discussion

In this study, we demonstrate that the plainfin midshipman can tolerate hypoxic stress for extended periods of time (>5 s). We suggest that this capacity is due to relatively low basal oxygen consumption rates, which they are able to adjust and markedly depress as a function of O_2 availability (fig. 1). Further, we propose that the glycolytic potential of parental males (i.e., glycogen reserves, metabolic enzymes) confers upon them an advantage over females when encountering a hypoxic stress. Finally, we attribute these differences between the sexes to both a body size advantage (allometry) and sex-specific tissue differences that provide an interesting example of biochemical sexual dimorphism with repercussions at the whole-organismal level.

Parental male *Poricthhys notatus* are sole providers of parental care during the reproductive season. They spend an extended period of time in the intertidal zone guarding the nests while the other reproductive morphs (females and sneaker males) leave shortly after fertilization (Hubbs 1920; Arora 1948; Brantley and Bass 1994). This environment is heavily impacted by the tidal schedule, as when the tide recedes, oxygen availability and other environmental factors can drastically change in the nests (Craig et al. 2014). Thus, we initially hypothesized that parental males would be more hypoxia resistant than their female counterparts. Indeed, when exposed to severe hypoxia, parental males were able to maintain responsiveness to a physical stimulus for almost twice as long as females. In a natural setting, this resistance would allow type I males to survive and remain alert even during prolonged low tides, whereas the females may become unresponsive to stimuli under these conditions, resulting in a potentially dangerous situation.

To further investigate these differences, we subjected individuals of both sexes to a gradual hypoxia challenge (fig. 1). In their reproductive state, normoxic *P. notatus* exhibited relatively low resting oxygen consumption rates when compared to other cold-adapted species (*P. notatus*, fig. 1, Craig et al. 2014; *Oncorhynchus mykiss*, Gonzalez and McDonald 1992; *Gadus morhua*, Lyndon et al. 1992). Low resting metabolic rates have been previously reported for other Batrachoidid species regardless of their reproductive status (*Opsanus beta*, Gilmour et al. 1998; *Opsanus tau*, Amorim et al. 2002). During the course of gradual hypoxia, animals of both sexes exhibited a similar reduction of oxygen consumption rates (fig. 1). As another proxy of metabolic activity, we evaluated the nitrogenous waste excretion rates of these fish during normoxia, hypoxia, and recovery (fig. 2). After an initial surge in urea excretion rate at the onset of hypoxia, the fish severely reduced their nitrogen excretion rates within 2 h of hypoxia, primarily through a reduction in ammonia excretion rate, and although fish rapidly recovered, they did not exhibit a compensatory response in nitrogen excretion rate (in the form of ammonia or urea) during the recovery period (fig. 2). This lack of compensation in nitrogen excretion rates further suggests that midshipman undergo metabolic depression under hypoxia (e.g., specifically in amino acid catabolism). Interestingly, the transient peak in urea excretion rate at the onset of hypoxia (fig. 2) coincided with the appearance of a mucoid substance containing urea (~0.34 mM; C. Bucking, unpublished observations) that was gastrointestinal in origin. Future research is required to determine the exact nature and purpose of these excretions.

We attempted to establish the critical oxygen tension (P_{crit})

Table 2: Metabolic enzyme activities in hypoxic and normoxic midshipman tissues

	Males		Females	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Gills:				
PK	45.91 ± 1.06[A]	49.03 ± 3.46[A]	29.24 ± 3.43[B]	33.58 ± 2.44[B]
LDH	53.15 ± 3.23	53.88 ± 5.93	47.44 ± 7.65	37.93 ± 7.85
CS	12.81 ± .62	13.51 ± .47	9.91 ± 1.80	12.41 ± 2.16
Skeletal muscle:				
PK	47.71 ± 7.28	53.85 ± 4.79	52.81 ± 4.09	40.01 ± 4.17
LDH	144.38 ± 17.62[A]	146.15 ± 14.90[A]	97.98 ± 8.51[B]	80.63 ± 6.78[B]
CS	3.08 ± .25	2.80 ± .36	2.68 ± .34	2.12 ± .35
Liver:				
PK	9.73 ± .40[A]	10.35 ± .77[A]	13.55 ± 1.00[B]	11.33 ± 2.11[B]
LDH	7.64 ± .52[A]	8.12 ± .42[A]	14.75 ± .74[B]	12.31 ± 1.72[B]
CS	.90 ± .07[A]	1.08 ± .08[A]	1.26 ± .13[B]	1.33 ± .22[B]

Note. Enzyme activities (in U/g tissue) were measured in tissues of parental male and female midshipman held under normoxia and after 3 h of gradual hypoxia exposure (see Material and Methods). Data are represented as means ± SEM. Different superscript letters indicate statistically significant differences between sexes (n = 4–6). PK = pyruvate kinase; LDH = lactate dehydrogenase; CS = citrate synthase.

at which *P. notatus* could not sustain its routine metabolic rate in the face of declining oxygen tensions. However, over the range of O_2 tension tested, the fish constantly adjusted their oxygen consumption as a function of oxygen availability (fig. 1), a characteristic of an oxyconformer. To date, only a few teleost species have been classified as oxyconformers, a situation in which organisms adjust their oxygen consumption to the oxygen tension in the environment (Hall 1929; Steffensen et al. 1982; Urbina et al. 2012). In *P. notatus*, this strategy would be advantageous, particularly for parental males that could rapidly adjust their metabolism to changing oxygen conditions. This capacity to oxyconform requires a suite of physiological adjustments enabling a reduction in metabolic demands and a switch in the biochemical pathways to produce energy (Hochachka 1986; Lutz and Storey 1997).

During hypoxia, there was an increase in hepatic glucose concentrations in both sexes but no significant change in hepatic glycogen depletion (table 1). Furthermore, regardless of the treatment, males exhibited elevated hepatic glycogen and glucose levels compared to females (table 1). These differences are exacerbated when these factors are corrected for the differential size of the liver in both sexes, as males have stores of glycogen and glucose that are 10–14 times higher than those of females (table 1). Further, even when accounting for sex-specific differences in body size, males still have more than seven times more glycogen and glucose reserves per gram of body mass than their female counterparts. In contrast, there were no corresponding changes in glucose, glycogen, or lactate levels in the skeletal muscle of parental males as reported previously (table 1; Craig et al. 2014). Hepatic glycogen reserves are heavily recruited in organisms during hypoxia, allowing glucose to be released in the blood and anaerobically used by other tissues (van den Thillart et al. 1980; van Waarde et al. 1983; Hochachka and Somero 1984). Thus, it appears that both males and females can mobilize glucose during hypoxia but that males have larger hepatic glycogen reserves that could enable them to sustain longer periods of hypoxic stress. In addition, these glycogen reserves may be of importance for parental males that are stranded in the nest for months with little opportunity to feed (Sisneros et al. 2009).

Surprisingly, when we looked at branchial NKA activity during hypoxia, it was unchanged in females and increased in parental males (fig. 3). These results seem at odds with an overall strategy to reduce energy expenditure under hypoxia. Indeed, NKA activity makes a large portion of the cellular energetic budget, and it would seem sensible to reduce its activity in an effort to depress energy consumption (Rolfe and Brown 1997). However, our NKA values are based on maximal ATP hydrolytic activity, an optimal situation where ATP is in excess. Thus, it is conceivable that, in vivo, ATP availability during hypoxia might be the factor limiting ATPase activity rather than its maximal capacity. This would explain, in part, the conflicting reports of changes in NKA activity in the gills of different hypoxic fish species (Richards et al. 2007; Woods et al. 2007; Garcia et al. 2008; Iftikar et al. 2010). In addition, an increased reliance on glycolytic and other anaerobic pathways to fuel ATPase activity in the gills could potentially reconcile an overall decrease in oxygen consumption and maintenance of osmoregulatory function in gills (e.g., Kultz and Somero 1995).

Previous work on midshipman suggested that field and laboratory hypoxia triggered an activation of anaerobic pathways (Craig et al. 2014). Further, females appear to rely less on glycolysis (using blood lactate as a proxy) than type I males when exposed to hypoxia (Craig et al. 2014). To explore the biochemical basis for these differences, we investigated activity levels of a mitochondrial marker (CS) and two glycolytic enzymes (LDH and PK) in midshipman tissues. These enzymes were unchanged in response to graded hypoxia (table 2). These results are not surprising considering the relatively short hyp-

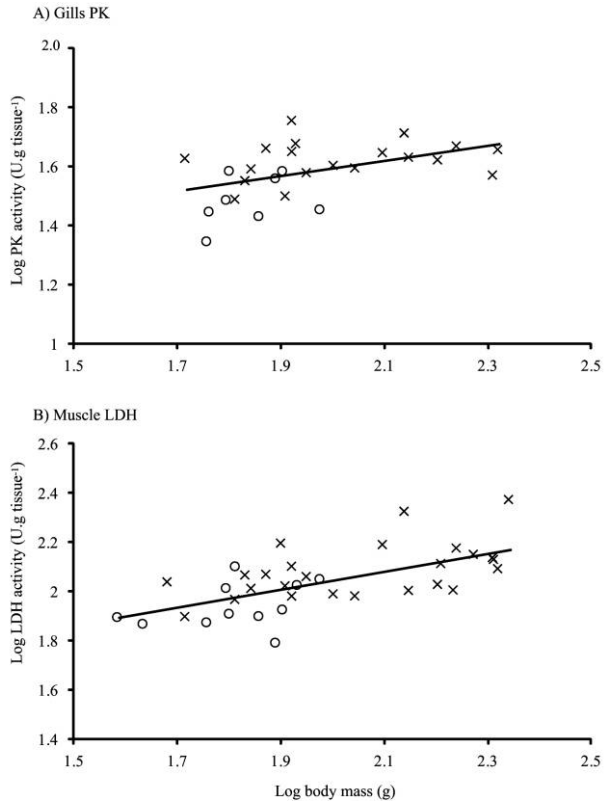

Figure 4. Relationships between body mass and enzyme activities in gills and skeletal muscle. The log-transformed values of body mass (g) and enzyme activities (U/g tissue) were plotted for pyruvate kinase (PK) in gills (*A*; $y = 0.26x + 1.08$, $r^2 = 0.22$) and lactate dehydrogenase (LDH) in muscle (*B*; $y = 0.37x + 1.31$, $r^2 = 0.40$), and a significant linear relationship was established ($n = 8$–11).

oxic bout, as enzymatic changes typically occur over longer hypoxia exposures (e.g., Greaney et al. 1980; Martinez et al. 2006). We further compared these enzyme activities between the sexes. Females had higher hepatic glycolytic enzyme activities per gram of tissue than males (table 2). Recall, however, that males have enlarged livers (see Material and Methods); thus, when corrected for whole-liver weight, the total hepatic activities of LDH and PK were similar in both sexes. In gills and skeletal muscles, the activity of the mitochondrial enzyme CS did not differ between sexes (table 2). There were higher levels of PK in male gills and of LDH in male muscles compared to females, suggesting sex-specific differences in the anaerobic capacities of these tissues (table 2). In several fish species, activities of glycolytic enzymes (e.g., LDH and PK) increase allometrically (Somero and Childress 1980; Almeida-Val et al. 2000). Thus, considering the larger body size of parental males compared to females, we explored the possibility that sex-specific enzymatic differences were driven by allometric relationships in *P. notatus*. There was a weak but positive relationship between gill PK activity and body mass (fig. 4*A*) and a more robust positive scaling exhibited by LDH activity in muscle and body mass (fig. 4*B*). Thus, it seems that in muscle and, to some extent, in gills, higher glycolytic enzyme activities may be dictated by body mass, as reported in other species (Somero and Childress 1980; Almeida-Val et al. 2000). However, given the weakness of the relationship, particularly for gill PK, we used the residuals from the linear relationships between mass-specific enzyme activities and body mass to assess whether sex had an influence on gill or muscle enzyme activities. Branchial PK, but not muscle LDH, was strongly influenced by the sex of the animal, with parental males having a significantly higher enzymatic activity than females. Overall, these results suggest that in *P. notatus*, sexual dimorphism both at the whole-organismal level (larger body size) and at the tissue level (enzyme activities) allows type I parental males to have higher glycolytic capacities in two energetically active tissues (gills and skeletal muscle). This higher glycolytic potential in males could provide a biochemical basis for the higher tolerance to severe hypoxia in the type I male *P. notatus* and may be of crucial importance for surviving the extreme conditions they encounter during the nesting season. Furthermore, our study provides an additional example of sex-specific divergent patterns of biochemical adaptations in Batrachoidid fish (Walsh et al. 1995).

Acknowledgments

We would like to thank the staff at the Bamfield Marine Sciences Centre for their help and cooperation during this study. In addition, we would like to thank Drs. Chris Wood and Grant McClelland for sharing equipment and chemicals instrumental to this work. This work was funded by the Canada Foundation for Innovation and a Natural Science and Engineering Research Council Discovery grant to P.J.W., who is also supported by the Canada Research Chair Program. P.M.C. was supported by a Natural Science and Engineering Research Council postdoctoral fellowship.

Literature Cited

Almeida-Val V.M., A.L. Val, W.P. Duncan, F.C. Souza, M.N. Paula-Silva, and S. Land. 2000. Scaling effects on hypoxia tolerance in the Amazon fish *Astronotus ocellatus* (Perciformes: Cichlidae): contribution of tissue enzyme levels. Comp Biochem Physiol B 125:219–226.

Amorim M.C.P., M.L. McCracken, and M.L. Fine. 2002. Metabolic costs of sound production in the oyster toadfish, *Opsanus tau*. Can J Zool 80:830–838.

Arora H.F. 1948. Observations on the habits and early life history of the batrachoid fish, *Porichthys notatus* Girard. Copeia 1948:89–93.

Bass A.H., D. Bodnar, and M.A. Marchaterre. 1999. Complementary explanations for existing phenotypes in an acoustic communication system. Pp. 493–514 in M.D. Hauser and M. Konishi, eds. The design of animal communication. MIT Press, Cambridge, MA.

Brantley R.K. and A.H. Bass. 1994. Alternative male spawning tactics and acoustic-signals in the plainfin midshipman fish

Porichthys notatus Girard (Teleostei, Batrachoididiae). Ethology 96:213–232.

Brauner C.J., C.L. Ballantyne, D.J. Randall, and A.L. Val. 1995. Air breathing in the armoured catfish (*Hoplosternum littorale*) as an adaptation to hypoxic, acidic, and hydrogen sulphide rich waters. Can J Zool 73:739–744.

Coleman R.M. 1999. Parental care in fishes. Pp. 165–180 in M.H. Horn, K.L.M. Martin, and M.A. Chotkowski, eds. Intertidal fishes: life in two worlds. Academic Press, New York.

Craig P.M., J.L. Fitzpatrick, P.J. Walsh, C.M. Wood, and G.B. McClelland. 2014. Coping with aquatic hypoxia: how the plainfin midshipman (*Porichthys notatus*) tolerates the intertidal zone. Environ Biol Fish 97:163–172.

Davenport J. and A.D. Woolmington. 1981. Behavioural responses of some rocky shore fish exposed to adverse environmental conditions. Mar Behav Physiol 8:1–12.

Davies R. and C.D. Moyes. 2007. Allometric scaling in centrarchid fish: origins of intra- and inter-specific variation in oxidative and glycolytic enzyme levels in muscle. J Exp Biol 210:3798–3804.

DeMartini E.E. 1988. Spawning success of the male plainfin midshipman. I. Influences of male body size and area of spawning site. J Exp Mar Biol Ecol 121:177–192.

Feder H.M., C.H. Turner, and C. Limbaugh. 1974. Observations on fishes associated with kelp beds in Southern California. Calif Dept Fish Game Fish Bull 160. 144 pp.

Garcia Sampaio F., C. de Lima Boijink, E. Tie Oba, L. Romagueira Bichara dos Santos, A. Lúcia Kalinin, and F. Tadeu Rantin. 2008. Antioxidant defenses and biochemical changes in pacu (*Piaractus mesopotamicus*) in response to single and combined copper and hypoxia exposure. Comp Biochem Physiol C 47:43–51.

Gawehn K. and H.U. Bergmeyer. 1974. Pp. 1492–1495 in H.U. Bergmeyer, ed. Methods of enzymatic analysis. Academic Press, London.

Gilmour K.M., S.F. Perry, C.M. Wood, R.P. Henry, P. Laurent, P. Pärt, and P.J. Walsh. 1998. Nitrogen excretion and the cardiorespiratory physiology of the gulf toadfish, *Opsanus beta*. Physiol Zool 71:492–505.

Gonzalez R.J. and D.G. McDonald. 1992. The relationship between oxygen consumption and ion loss in a freshwater fish. J Exp Biol 163:317–332.

Greaney G.S., A.R. Place, R.E. Cashon, G. Smith, and D.A. Powers. 1980. Time course of changes in enzyme activities and blood respiratory properties of killifish during long-term acclimation to hypoxia. Physiol Zool 53:136–144.

Hall F.G. 1929. The influence of varying oxygen tensions upon the rate of oxygen consumption in marine fishes. Am J Physiol 88:212–218.

Hochachka P.W. 1986. Defense strategies against hypoxia and hypothermia. Science 23:234–241.

Hochachka P.W. and G. Somero. 1984. Biochemical adaptation. Princeton University Press, Princeton, NJ.

Hubbs C.L. 1920. The bionomics of *Porichthys notatus* Girard. Am Nat 54:380–384.

Iftikar F.I., V. Matey, and C.M. Wood. 2010. The ionoregulatory responses to hypoxia in the freshwater rainbow trout *Oncorhynchus mykiss*. Physiol Biochem Zool 83:343–355.

Ivancic I. and D. Degobbis. 1984. An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. Water Res 18:1143–1147.

Jungmann R.A., D. Huang, and D. Tian. 1998. Regulation of *LDH-A* gene expression by transcriptional and posttranscriptional signal transduction mechanisms. J Exp Zool 282:188–195.

Kultz D. and G.N. Somero. 1995. Ion transport in gills of the euryhaline fish *Gillichthys mirabilis* is facilitated by a phosphocreatine circuit. Am J Physiol 268:R1003–R1012.

LeMoine C.M., C.E. Genge, and C.D. Moyes. 2008. Role of the PGC-1 family in the metabolic adaptation of goldfish to diet and temperature. J Exp Biol 211:1448–1455.

Llorente P., R. Marco, and A. Sols. 1970. Regulation of liver pyruvate kinase and the phosphoenolpyruvate crossroads. Eur J Biochem 13:45–54.

Lutz P.L. and K. Storey. 1997. Strategies for dealing with variations in gas tensions vertebrates and invertebrates. Pp. 1479–1522 in W. Danzler, ed. Handbook of physiology. Vol. 13. Comparative physiology. Oxford University Press, Oxford.

Lyndon A.R., D.F. Houlihan, and S.J. Hall. 1992. The effect of short-term fasting and a single meal on protein synthesis and oxygen consumption in cod, *Gadus morhua*. J Comp Physiol B 162:209–215.

Mandic M., K.A. Sloman, and J.G. Richards. 2009. Escaping to the surface: a phylogenetically independent analysis of hypoxia-induced respiratory behaviors in sculpins. Physiol Biochem Zool 82:703–738.

Martin K.L.M. 1993. Aerial release of CO_2 and respiratory exchange ratio in intertidal fishes out of water. Environ Biol Fish 37:189–196.

———. 1996. An ecological gradient in air-breathing ability among marine cottid fishes. Physiol Zool 69:1096–1113.

Martinez M.L., C. Landry, R. Boehm, S. Manning, A.O. Cheek, and B.B. Rees. 2006. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the gulf killifish, *Fundulus grandis*. J Exp Biol 209:3851–3861.

McClelland G.B., A.C. Dalziel, N.M. Fragoso, and C.D. Moyes. 2005. Muscle remodeling in relation to blood supply: implications for seasonal changes in mitochondrial enzymes. J Exp Biol 208:515–522.

McCormick S.D. 1993. Methods for the nonlethal gill biopsy and measurements of Na^+,K^+-ATPase activity. Can J Fish Aquat Sci 50:656–658.

Morris S. and A.C. Taylor. 1983. Diurnal and seasonal variation in physico-chemical conditions within intertidal rock pools. Estuar Coast Shelf Sci 17:339–355.

Muñoz M.E. and E. Ponce. 2003. Pyruvate kinase: current status of regulatory and functional properties. Comp Biochem Physiol B 135:197–218.

Muusze B., J. Marcon, G. van den Thillart, and V.M.F. Almeida-Val. 1998. Hypoxia tolerance of Amazon fish: respirometry

and energy metabolism of the cichlid *Astronotus ocellatus*. Comp Biochem Physiol A 120:151–156.

Passonneau J.V. and V.R. Lauderdale. 1974. A comparison o three methods of glycogen measurement in tissues. Anal Biochem 60:405–412.

Rahmatullah M. and T.R. Boyde. 1980. Improvements in th determination of urea using diacetyl monoxime: methods with and without deproteination. Clin Chim Acta 107:3–9.

Richards J.G., Y.S. Wang, C.J. Brauner, R.J. Gonzalez, M.L Patrick, P.M. Schulte, V.M. Chippari-Gomes, V.M.F. Almeida-Val, and A.L. Val. 2007. Metabolic and ionoregulatory responses of the Amazonian cichlid, *Astronotus ocellatus*, t severe hypoxia. J Comp Physiol B 177:361–374.

Rolfe D.F.S. and G.C. Brown. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals Physiol Rev 77:731–758.

Sisneros J.A., P.W. Alderks, K. Leon, and B. Sniffen. 2009. Morphometric changes associated with the reproductive cycle and behaviour of the intertidal-nesting, male plainfin midshipman *Porichthys notatus*. J Fish Biol 74:18–36.

Somero G.N. and J.J. Childress. 1980. A violation of the metabolism size scaling paradigm: activities of glycolytic enzymes in muscle increase in larger-size fish. Physiol Zool 53: 322–337.

Steffensen J.F., J.P. Lomholt, and K. Johansen. 1982. Gill ventilation and O_2 extraction during graded hypoxia in two ecologically distinct species of flatfish, the flounder (*Platichthys flesus*) and the plaice (*Pleuronectes platessa*). Environ Biol Fish 7:157–163.

Truchot J.-P. and A. Duhamel-Jouve. 1980. Oxygen and carbon dioxide in the marine intertidal environment: diurnal and tidal changes in rockpools. Respir Physiol 39:241–254.

Urbina M.A., C.N. Glover, and M.E. Forster. 2012. A novel oxyconforming response in the freshwater fish *Galaxias maculatus*. Comp Biochem Physiol A 161:301–306.

van den Thillart G., F. Kesbeke, and A. van Waarde. 1980. Anaerobic energy-metabolism of goldfish, *Carassius auratus* (L.). J Comp Physiol 136:45–52.

van Waarde A., G. van den Thillart, and F. Kesbeke. 1983. Anaerobic energy metabolism of the European eel, *Anguilla anguilla*. Comp Physiol 149:469–475.

Walker H.J., Jr., and R.H. Rosenblatt. 1988. Pacific toadfishes of the genus *Porichthys* (Batrachoididae) with descriptions of three new species. Copeia 1988:887–904.

Walsh P.J., T.P. Mommsen, and A.H. Bass. 1995. Biochemical and molecular aspects of singing in Batrachoidid fishes. Pp. 279–289 in P.W. Hochachka and T.P. Mommsen, eds. Biochemistry and molecular biology of fishes. Vol. IV. Metabolic and adaptational biochemistry. Elsevier, New York.

Wood C.M., M. Kajimura, K.A. Sloman, G.R. Scott, P.J. Walsh, V.M.F. Almeida-Val, and A.L. Val. 2007. Rapid regulation of Na^+ fluxes and ammonia excretion in response to acute environmental hypoxia in the Amazonian oscar, *Astronotus ocellatus*. Am J Physiol 292:R2048–R2058.