

Coping with aquatic hypoxia: how the plainfin midshipman (*Porichthys notatus*) tolerates the intertidal zone

Paul M. Craig · John L. Fitzpatrick · Patrick J. Walsh ·
Chris M. Wood · Grant B. McClelland

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Abstract Although plainfin midshipman (*Porichthys notatus*) are primarily known for their alternative reproductive tactics, and the dimorphic male subtypes, in which Type-I males demonstrate parental investment and mate attraction, and Type-II males ‘sneak’ fertilization and show no investment after fertilization, little is known about the physiology and tolerance to low aquatic oxygen while nesting in the intertidal zone. In May 2007, females and Type-I and Type-II males were collected, and in June 2009, only Type-I males were collected from nest sites on the coast of Vancouver Island, British Columbia. In the 2007 season, an initial assessment of hypoxia tolerance and nest parameters was recorded for the three subtypes of midshipman. Historical evidence indicates that Type-I males remain on the nest for prolonged periods,

and our results suggest they can cope with repeated bouts of aquatic hypoxia by elevating their hematocrit and tolerating high lactate levels. The 2009 season was directed at examining the aquatic hypoxia tolerance of only the Type-I male. Hypoxic (~15 % air saturated water) Type-I males had oxygen consumption rates at ~12 % of the normoxic control (~100 % air saturated water) and a P_{crit} , the critical oxygen tension, when a fish switches from oxyregulator to oxyconformer, could not be determined; an indication that these fish are solely oxyconformers. With prolonged exposure to aquatic hypoxia, Type-I males displayed significant elevations in plasma and tissue lactate (heart), tissue glucose (liver), and a depression in gill Na^+/K^+ ATPase and catalase activities. Results suggest that male Type-I midshipman survival in the

P. M. Craig · J. L. Fitzpatrick · C. M. Wood ·
G. B. McClelland
Department of Biology, McMaster University,
1280 Main St W,
Hamilton, Ontario, Canada L8S 4K1

P. J. Walsh
Department of Biology, University of Ottawa,
30 Marie Curie,
Ottawa, Ontario, Canada K1N 6N5

P. M. Craig · J. L. Fitzpatrick · P. J. Walsh · C. M. Wood ·
G. B. McClelland
Bamfield Marine Sciences Centre,
100 Pachena Drive,
Bamfield, British Columbia, Canada V0R 1B0

J. L. Fitzpatrick
Computational and Evolutionary Biology,
Faculty of Life Sciences, University of Manchester,
Manchester M13 9PT, UK

Present Address:
P. M. Craig (✉)
Department of Biology, University of Ottawa,
30 Marie Curie,
Ottawa, Ontario, Canada K1N 6N5
e-mail: pcraig@uottawa.ca

intertidal zone is enhanced by metabolic depression and tolerance to anaerobic byproducts.

Keywords Aquatic hypoxia · Plainfin midshipman · Marine · Intertidal

Introduction

Plainfin midshipman (Family Batrachoididae; *Porichthys notatus* Girard 1854) are distributed along a large ecological niche on the Pacific coast of North America, ranging from the Baja Peninsula to Alaska (Walker and Rosenblatt 1988). During the breeding season (May–August; Vancouver Island, BC), midshipman migrate from depth and take up residence in the intertidal zone where females are courted by vocalizations of males (called Type I males) who have established nest sites underneath large rocks (Arora 1948; Thompson et al. 1988; Bass et al. 1999). After male fertilization of the egg mass, the female departs the nest, leaving the Type I male to care for the eggs. Oviposition on the underside of intertidal rocks is key to the survival of the eggs and larvae during low tide and air emergence, and survival is enhanced by the parental care of the male which consists of gentle fanning, cleaning the nest site, and hydrating the eggs if they dry during low tide (Arora 1948). Once hatched, the larvae remain attached to the underside of the rock by an adhesive disc, all while the Type I male remains on the nest to ensure safety for the larvae until they depart as juveniles, approximately 45 days post-fertilization (Arora 1948). Parental care in this taxon is unique, as the plainfin midshipman is the only known intertidal fish that provides care beyond the point of hatching (Coleman 1999). Interestingly, male midshipman fish are dimorphic. While Type-I males invests in vocalization for mate attraction, territorial defense, and parental care, another type of male that are much smaller in comparison, called Type-II males, may steal fertilization from Type-I males using female mimicry or sneaking behaviors (Brantley et al. 1993; Brantley and Bass 1994). Type-II males and females do not remain on the nest sites for long periods of time (Brantley and Bass 1994). Their reproductive behaviours have identified midshipman as a target for many studies directed at alternative reproductive tactics (e.g., Brantley and Bass 1994), but little is known regarding the basic physiology of these fish. More

specifically, we are aware of no studies on the responses to aquatic hypoxia of the nesting Type-I males, who must remain on the nest site for extended periods of time.

Fish that reside in the intertidal zone are faced with the cyclic environmental stressor of hypoxia during the daily oscillations of the tide. Those that do not retreat with the receding waters of low tide are trapped in shallow pools that are invariably depleted of oxygen and need to invoke a variety of protective physiological or behavioral responses. This is especially true for the plainfin midshipman. During low tide, these fish (particularly the Type-I males) are often stranded in nests, which are found beneath large rocks in the intertidal flats, with significantly diminished dissolved oxygen levels (lowest personal observation: 2.5 % air saturated water) or nests devoid of water (personal observation), for several hours where they must endure dramatic reductions in available oxygen content (Martin and Bridges 1999). Through a combination of the physicochemical properties of oxygen solubility in seawater, drastic temperature changes, decreases in pH and salinity in intertidal pools, organismal respiration and daytime photosynthesis, a rapid depletion of oxygen may occur once the tide has receded (Truchot and Duhamel-Jouve 1980; Bridges 1993).

Recently, Perry et al. (2010) examined the acid–base regulation of plainfin midshipman, and demonstrated that they have a well-developed ability to tolerate severe hypercapnic exposure (5 % water CO₂), which also may occur in the aquatic environment of nesting fish. This is accomplished by increasing plasma HCO₃⁻, although clearance of HCO₃⁻ when returned to normocapnia (<0.1 % water CO₂) was rather slow, possibly the consequence of an aglomerular kidney (McDonald and Walsh 2007). In addition, we have documented that the sperm of this species is well adapted to function under aquatic hypoxic conditions (Fitzpatrick et al. 2009). However, to date, no study has examined the physiology of the midshipman fish itself under aquatic hypoxia. The objectives of the present study are to make an initial assessment of the ability of the plainfin midshipman to tolerate daily bouts of aquatic hypoxia in the intertidal zone, with comparison made between females and Type II males. Additionally, Type-I males were collected and subjected to progressive hypoxia, and measurements of oxygen consumption were taken for identification of midshipman being either an

oxyregulator (constant respiratory rate under declining water oxygen tensions) or oxyconformer (respiratory rate declines in parallel with reduced oxygen tensions). Further analysis of plasma, tissue metabolites, and enzyme activities were taken to measure their tolerance of anaerobic by-products.

Materials and methods

Field data

Field collection Plainfin midshipman were collected from nest sites on Vancouver Island, British Columbia, under permits from the Department of Fisheries and Oceans, Canada. All experiments were conducted under protocols approved by the McMaster University Animal Research Ethics Board and conform to the principles of the Canadian Council for Animal Care. To avoid impacts on the population, numbers were kept to a minimum while retaining statistical power. Collections of both sexes were first made during low tide in late May of 2007 from Mill Bay (48°63'N, 123°53'W) for field measurements of hematocrit (Hct) and plasma lactate (Type-I males, $n=11$, 216.6 ± 34.3 g; females, $n=12$, 120 ± 17.3 g; Type-II males, $n=7$, 80.5 ± 12.8 g). Male morphs were determined based on behavioural observations in the field (only Type I males guard nests), body size and morphological differences, which included extreme differences in body mass and length (see Brantley and Bass 1994), differences in gonadosomatic index and presence of a large sonic muscle in Type-I males. Upon capture from nests, a caudal blood sample was taken and centrifuged immediately for Hct and plasma was stored in liquid nitrogen for later analysis of lactate. In early June of 2009 only Type-I males ($n=16$; 171.1 ± 15.8 g, morph determined as outlined above) were collected from Nanoose Bay (49°15'N, 124°10'W) for laboratory experimentation of progressive hypoxia. Nest site parameters (temperature, pH, dissolved O₂) were recorded during the peak of low tide (June 6th 2009, 11:40 am) using a calibrated-handheld Accumet pH/dissolved oxygen meter (Fisher Scientific). Additional water samples were taken for measurement of urea and ammonia, which were quantified using the diacetyl monoxime method of Rahmatullah and Boyde (1980) and indophenol blue method of Ivancic and Degobis (1984), respectively. Fish were transported in

aerated seawater baths and housed at Bamfield Marine Sciences Centre in aerated tanks (10 °C) with a continuous renewal of fresh seawater that was pumped directly from Bamfield Inlet into the holding tanks. Tanks [0.25 m (h)×0.6 m (w)×2.5 m (l)] were supplied with large rocks and PVC piping which provided shelter, in addition to a large tarpaulin that covered the tanks to minimize disturbance. These animals were not fed during the duration of the experiments, as Type-I males do not feed while on the nest, and therefore fast during the period of parental care (Coleman 1999). Fish were housed for a period of 1 week prior to experimentation.

Laboratory data

Progressive hypoxia Individual fish (Type-I males) were placed in darkened 1.5-L Mason jars (1.4 L water) and allowed to adjust for 1 h with a continuous flow of normoxic seawater. Seawater flow was stopped and the jars were sealed with a modified rubber stopper. All jars were bathed in free-flowing 10 °C seawater, to keep temperature within the jars constant. An initial 0.5-mL water sample and additional 0.5-mL samples were taken every 30 min for a total exposure time of 3 h through the rubber stopper using a 1-mL syringe. Water oxygen tension was determined immediately after sampling using a Radiometer-Copenhagen O₂ electrode maintained at experimental temperature. Oxygen consumption rates (MO₂) were determined over each 30 min period according to the change in the dissolved oxygen in the chamber multiplied by the water volume, and expressed relative to the body mass of the fish. P_{crit}, or the critical oxygen tension at which the fish is considered an oxyconformer, was determined using the least squares regression method outlined by Hastings and Burggren (1995), based on the computer model described by Yeager and Ultsch (1989).

Hypoxic exposure and tissue sampling After a week of recovery in their normoxic holding tanks, the Type-I males, which had been used for the progressive hypoxia experiment, were now employed for an assessment of tissue metabolites during hypoxia. These fish were placed in 6 L of seawater in polyethylene Omni Boxes™ (Canadian Tire Corp, Canada) bubbled with either air (normoxic, 100 % air saturated water; $n=8$) or nitrogen (hypoxic, 15 % air saturated water; $n=8$) for

4 h to simulate intertidal oxygen deprivation (Table 1). The Omni Boxes™ were bathed in a continuous flow of seawater to ensure temperature was maintained at approximately 10 °C. At the end of the exposure, fish were removed and terminally anesthetized rapidly with a lethal overdose of tricaine methanesulfonate (1.0 g/L MS-222, Sigma; neutralized with 1.0 g/L NaHCO₃), immediately followed by a caudal blood sample for measurements of Hct and lactate, and tissue harvesting of gill, liver and heart, which were rinsed with saline to removed residual blood, and freeze-clamped in liquid nitrogen for later analysis of metabolite concentrations and enzyme activities.

Enzymatic and metabolite assays Unless noted, all chemicals and reagents were purchased through Sigma (St Louis, USA). Heart, liver, and gill from normoxia and hypoxia exposed fish were powdered using a liquid nitrogen-cooled motor and pestle for measurements of tissue glucose, glycogen and lactate. Powdered tissues were weighed, freeze-dried over night (Labconco Freeze dry systems), and 50 mg of dried-powdered tissue was then homogenized in 20-times of volume extraction buffer containing 20 mM Hepes (pH 7.0), 1 mM EDTA, and 0.1 % Triton X-100 for quantification of enzymatic activity in the liver and gill. Samples for tissue metabolites (glucose, glycogen, and lactate) were homogenized in 2 volumes 8 % perchloric acid containing 1 mM EDTA, and samples were split and neutralized with either 2 M KOH for lactate or 3 M K₂CO₃ for glucose and glycogen samples. Tissue glucose, glycogen, lactate and plasma lactate were measured at 340 nm following standard spectrophotometric protocols (Bergmeyer 1983). For enzyme assays, gill and liver tissues were homogenized in ice-cold 50 mM phosphate buffer (50 mM KH₂PO₄, 50 mM K₂HPO₄), containing 1 mM EDTA, pH 6.7. Catalase (CAT) activity was assayed at 28 °C using a modified method based on Clairborne (1985). The reaction buffer consisted of 20 mM potassium phosphate, pH 7.0, and 20 mM H₂O₂. The reaction was recorded as the decomposition of H₂O₂ at 240 nm over 1 min. Protein concentrations for all samples were determined using BioRad kit reagents following the Bradford (1976) protocol using bovine serum albumin as a standard. Tissue Na⁺/K⁺-ATPase activity (NKA) was determined using the microassay method of McCormick (1993). All samples were homogenized in SEI buffer (150 mM

sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) containing 0.3 % Na deoxycholic acid, which acts as an ionic detergent in the cell lysis buffer for membrane bound protein isolation. All assays were run in 96-well format on a SpectraMAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA).

Statistical analysis Data have been expressed as mean ± S.E.M. A one-way ANOVA was used to ascertain the overall differences for Hct and plasma lactate from field samples, with a Student-Newman-Keuls (SNK) post hoc test for all pairwise comparisons. A repeated measure ANOVA on ranks was used to assess the overall differences during progressive hypoxia, as normality assessment failed, with a Holm-Sidak post hoc test for pairwise comparisons to the normoxic control. A Student's *t*-test was used for all other pairwise analysis. All statistical analyses were performed using Sigma Stat (v3.1, Systat Software, Inc.) with a significance level of $p < 0.05$.

Results

Nest parameters Dissolved oxygen levels recorded during the peak of low tide from nest sites ($n=8$) found in Nanoose Bay in 2009 were 85 % lower than open water values (Table 1), which gave us an estimate of the maximum average depletion of oxygen in the nests to use as laboratory conditions for further hypoxic experiments (see below). Along with reductions in dissolved oxygen there was a significant ~4-fold increase in water ammonia, and a ~2-fold increase in

Table 1 Water parameters of pH, temperature (°C), oxygen saturation (%), ammonia and urea (μmol/L) taken during the peak low tide (11:40 am) from midshipman nest sites ($n=16$) on Nanoose Bay on June 6th, 2009

Parameter	Nest	Open water
pH	7.91±0.2*	8.45±0.1
°C	19.63±0.4	19.6±1.2
Oxygen saturation (%)	14.5±4.1 *	100±3.2
Ammonia	14.98±1.9*	3.92±0.2
Urea	14.81±7.1*	7.71±0.8

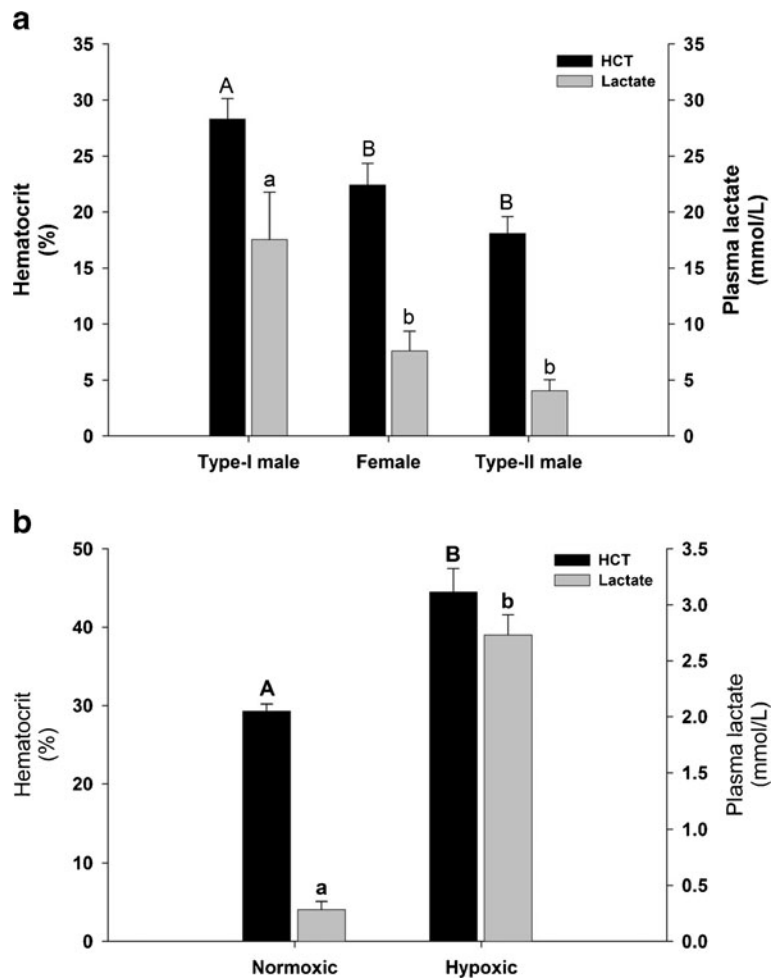
A * indicates a significant difference as compared to open water values taken for the intertidal zone at low tide on the same day ($p < 0.05$)

water urea (Table 1). The seawater nest sites were slightly acidified, with a significant decrease in mean pH from 8.4 in open ocean waters to 7.9 (Table 1). It is noted that temperature in both the nests and adjacent bulk water of the embayment were significantly higher than historical seawater temperatures (~19 °C vs ~10–13 °C) in the area, due to unseasonably warm (28 °C air temperature) weather combined with a flat-graded intertidal zone (very shallow, without a sharp drop-off) which likely elevated the shore water temperature.

Enzymatic and metabolite response to hypoxia Field measurements of both hematocrit (ANOVA; $P=0.004$) and plasma lactate (ANOVA; $P=0.012$) were significantly lower in Type-II males (SNK for Hct: $P=0.004$; lactate: $P=0.018$) and females (SNK for Hct: $P=0.025$; lactate: $P=0.018$) than Type-I males (Fig. 1a). In a laboratory setting, there was an inverse relationship

between ambient oxygen tension and both Hct ($P=0.002$) and lactate ($P<0.001$) in midshipman exposed to normoxic (95.7 ± 3.0 % air saturated water) or hypoxic (14.5 ± 2.9 % air saturated water) conditions for 4 h (Fig. 1b). However, we did not observe the same absolute magnitude of plasma lactate increase in the laboratory as seen in the field data collected, as the mean plasma lactate in Type-I males exposed to hypoxia in the laboratory was less than 3.0 mmol L^{-1} , or ~6-fold lower than values obtained from Type-I males in the field in 2007 (Fig. 1). Fish exposed to hypoxia in the laboratory showed no changes in tissue lactate in the liver or muscle, yet there was a significant 4-fold increase in tissue lactate in the heart (Fig. 2a; $P=0.036$). Note that these metabolite concentrations (Fig. 2) were expressed per unit-dry weight, whereas plasma lactate was expressed per unit

Fig. 1 **a** Hematocrit (%) and plasma lactate (mmol/L) values from Type-I ($n=11$), -II ($n=7$), and female ($n=12$) midshipman fish collected in May 2007 at Mill Bay, Vancouver Island, British Columbia. **b** Hematocrit (%) and plasma lactate values (mmol/L) from Type-I males collected June 2009 from Nanoose Bay and exposed to either normoxic (~100 % air saturated water; $n=8$) or hypoxic (~15 % air saturated water, $n=8$) conditions in the laboratory. Bars that do not share common letter are significantly different from each other



volume (Fig. 1). Although there were no changes in glycogen levels across all tissues, there was a 4.6-fold increase in liver glucose levels (Fig. 2b and c; $P < 0.001$). Measurements of catalase and

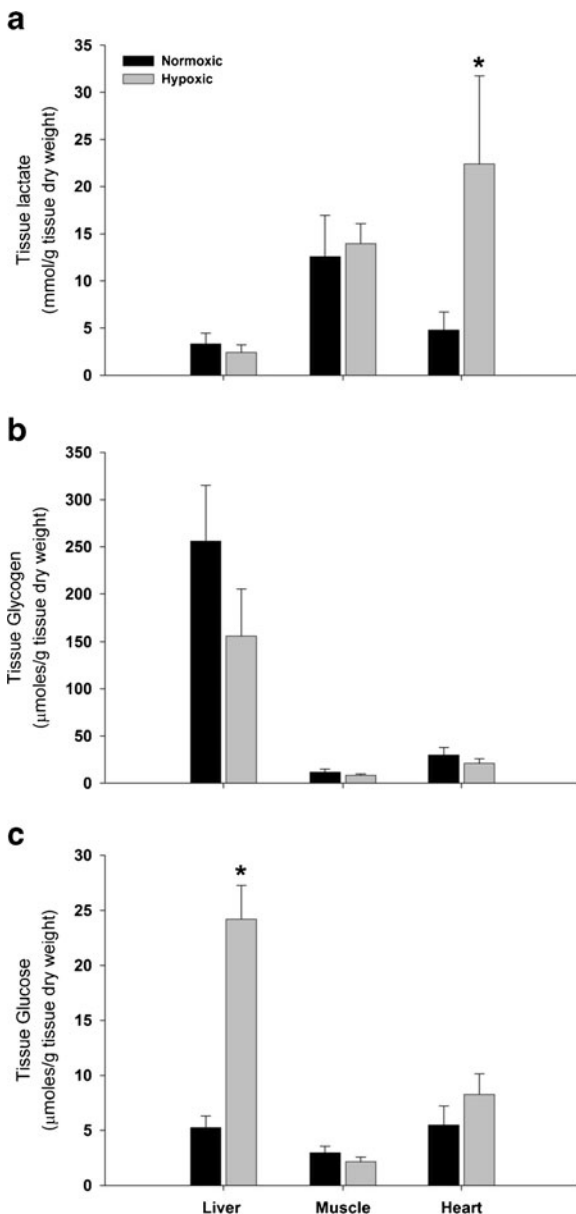


Fig. 2 **a** Liver, muscle, and heart tissue lactate ($\mu\text{mol/g}$ tissue dry weight), **b** glycogen ($\mu\text{mol/g}$ tissue dry weight), and **c** glucose ($\mu\text{mol/g}$ tissue dry weight) values taken from Type-I males collected June 2009 from Nanoose Bay exposed to either normoxic ($\sim 100\%$ air saturated water; $n=8$) or hypoxic ($\sim 15\%$ air saturated water, $n=8$) conditions in the laboratory. An *asterisk* indicates a significant difference between normoxic and hypoxic conditions within a tissue (t -test, $p < 0.05$)

NKA activities were taken as indicator of cellular oxygen stress and energy consumption, respectively. Although there were no apparent changes for either enzyme in the liver of hypoxia-exposed fish, there were significant reductions in gill enzymatic activity for NKA ($\sim 40\%$; Fig. 3a; $P=0.032$) and catalase ($\sim 50\%$; Fig. 3b; $P=0.047$) in hypoxia-exposed fish compared to the normoxic controls.

Progressive hypoxia and metabolic rate Midshipman (Type-I males) significantly decreased overall oxygen consumption during progressive hypoxia in an oxyconformer fashion, as P_{crit} could not be determined for this species. Linear regression analysis revealed a significant positive relationship between water oxygen tension and oxygen consumption (Fig. 4. $R^2=0.714$, $P < 0.001$).

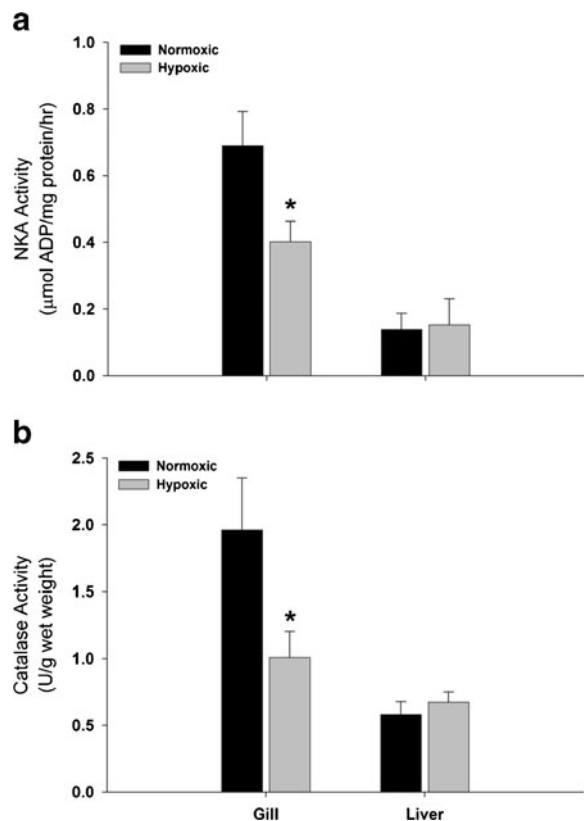


Fig. 3 **a** Enzyme activities levels for Na^+K^+ ATPase and **b** catalase from gill and liver tissue sampled from Type-I males collected June 2009 from Nanoose Bay exposed to either normoxic ($\sim 100\%$ air saturated water; $n=8$) or hypoxic ($\sim 15\%$ air saturated water, $n=8$) conditions in the laboratory. An *asterisk* indicates a significant difference between normoxic and hypoxic conditions within a tissue (t -test, $p < 0.05$)

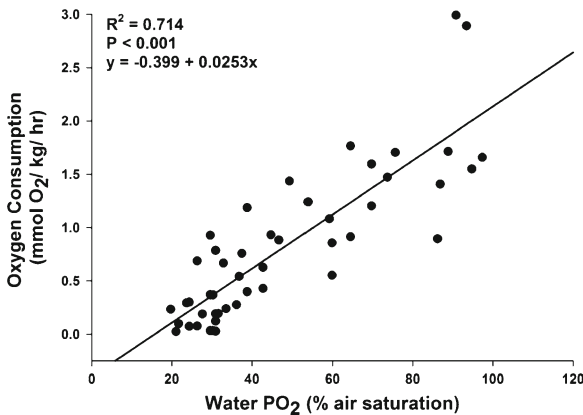


Fig. 4 The significant linear relationship between oxygen consumption versus % air saturation in water of male Type-I midshipman during progressive hypoxia. Using the least squares regression method outlined by Hastings and Burggren (1995) a P_{crit} could not be determined and therefore these fish were identified as oxyconformers. ($R^2=0.714$; $P<0.001$)

Discussion

This study provides unique insight into aquatic hypoxia tolerance associated with alternative reproductive tactics, as displayed through the differential buildup of anaerobic byproducts in females and male subtypes (Fig. 1a). Our results demonstrate the Type-I morph, the parental care provider, is able to survive extended (3–4 h) hypoxic conditions through increased anaerobic glycolysis (Fig. 2), metabolic suppression (Figs. 3 and 4), and tolerance to anaerobic byproducts (Fig. 1). Notably, these animals are fasting during this period of parental care, and all these responses must be superimposed on the responses to extended food deprivation. Evidence for this is the routine MO₂ measured by Walsh et al (2001) in freshly trawled midshipman having an average oxygen consumption of ~2.5 mmol O₂ kg⁻¹ h⁻¹ vs the present study with ~1.0 mmol O₂ kg⁻¹ h⁻¹ (Fig. 4).

As first observed by Hubbs (1920) and later corroborated by Arora (1948), it is the Type I male midshipman who remains on the nest and provides parental care and protection to the developing embryos, only to leave once the juveniles are free-swimming. The females reside in the nest for only a short period of time, and vacate soon after oviposition takes place. Furthermore, there are two male morphs, Type-I males (who show parental investment) and Type-II (“sneakers” who show no investment after fertilization; Brantley et al. 1993;

Brantley and Bass 1994). Like the females, Type-II males tend to retreat from the nests once fertilization has occurred, as both are difficult to locate within a nest soon after the initiation of the breeding season (Fitzpatrick 2008). Early in the breeding season, many nest sites hold females and both male morphs, unlike later in the season when only Type-I males are on nest guarding developing embryos. Furthermore, from field samples taken in 2007, we demonstrated that the Type-I males have significantly higher values for both Hct and plasma lactate (Fig. 1a), likely due to the length of time the Type-I morph spent on the nest compared to the female and Type-II male. Increased Hct has been linearly associated with increased oxygen carrying capacity in some studies (Baldwin and Wells 1990; Sadler et al. 2000), and it has been suggested that changes in carrying capacity of blood are more important than alterations in blood oxygen affinity to deliver oxygen to tissues (Brauner and Wang 1997). It appears an initial physiological response to hypoxia is increased Hct, as seen both in the wild and laboratory experiments (Fig. 1). Additionally, we see greatly elevated plasma lactate values taken from field-collected midshipman, suggesting increased reliance on anaerobic metabolism associated with hypoxia (Fig. 1a). This lactate elevation was confirmed in our laboratory-simulated hypoxia experiment, yet the magnitude of the response was lower than that obtained from field results. There can be several explanations for this discrepancy. It is common for field lactate concentration values to be higher than laboratory measurements, as been demonstrated in both reptiles (Bennett et al. 1981) and fish species (Scholander et al. 1962; Martin et al. 2004). The elevated lactate values seen here suggest that anaerobic metabolism may significantly contribute to the daily activity of the fish. When considering the territorial nature of the Type-I male when guarding a nest, it is plausible that field measurements represent vigorous exertion, particularly during capture, is likely fueled anaerobically. Additionally, the capture method and exertion during sampling may have resulted in higher than expected lactate levels, as it is well documented in Rainbow trout that duration of handling rapidly increases plasma cortisol, lactate, and glucose (Meka and McCormick 2005). However, our handling times were short and efforts were made to ensure caudal blood

sampling consistently occurred within 2 min of capture in both males and females. Nevertheless, with the significant difference between females and males morphs associated with plasma lactate and Hct, our data suggest that male Type-I midshipman are adapted for continuous bouts of hypoxia, as they have a potential for greater oxygen carrying capacity and tolerance to a build-up of plasma lactate.

Male Type-I midshipman have low oxygen consumption rates while normoxic, similar to that of other hypoxia-tolerant fish species (see Martin and Bridges 1999), and further decrease their oxygen consumption to ~12 % of their normoxic rate under hypoxic conditions (Fig. 4). However, a critical oxygen tension at which midshipman switch from oxyregulation to oxyconformation (P_{crit} ; Pörtner and Grieshaber 1993) was not evident; an indication that midshipman are oxyconformers. As Scott et al. (2008) suggest, a defined P_{crit} is not an essential marker for hypoxia tolerance, and in the case of midshipman, being an oxyconformer may be an adaptive response to daily cycles of aquatic hypoxia. To conserve energy, it would be advantageous to conform to ambient oxygen concentrations and rapidly reduce metabolism during the initial onset of hypoxic conditions, as opposed to expending energy regulating oxygen consumption by behavioral or physiological means. In the case of the plainfin midshipman, decreased oxygen consumption (Fig. 4) and reduced ATP demand (e.g. decreased gill Na^+K^+ ATPase (NKA) activity; Fig. 3) provide evidence in favor of this adaptive response, although we cannot rule out down-regulated gill enzymatic activity was due to air emergence. Active proton pumping across the plasma membrane is a major consumer of mitochondrial ATP production, where NKA consumes anywhere between 4 % and 50 % of basal energy production depending on the tissue examined (Clausen et al. 1991). Further examination of arterial blood gases (O_2 , CO_2) during hypoxia exposure are necessary and would assist in further elucidating the hypoxia tolerance of midshipman.

It is well established that under hypoxic conditions, tolerant fish will resort to metabolic suppression and increased reliance on anaerobic metabolism and glycolytic pathways (Hochachka et al. 1996; Guppy and Withers 1999; Bickler and Buck 2007). The present results demonstrated that male Type-I midshipman had increased plasma lactate (Fig. 1b), and changes

in liver glucose and heart lactate concentrations after a 4-h aquatic hypoxic exposure (Fig. 2), although there were no statistical decreases in liver glycogen content. However, in two different Amazonian species exposed to 8 h of graded hypoxia, liver glycogen decreased; an indication of increased glycogenolysis (Chippari-Gomes et al. 2005). In relation to our study, the length of hypoxic exposure (4 h) may not have been long enough to produce a statistical decrease in liver glycogen content. Nevertheless, the increase in glucose in the liver is an indication of increased glycogenolysis for glucose release into the circulation, which may fuel anaerobic glycolytic pathways in other tissues; a resulting effect is the generation of lactate, as noted in both the plasma and tissues, specifically the heart. Lactate produced in these tissues under hypoxic conditions may be stored until favorable conditions return, and anaerobic byproducts metabolized. Our evidence suggests that the strategy used by parental midshipman to tolerate hypoxia is maintenance of ATP homeostasis through a combination of reduction in ATP-consuming processes (e.g. NKA activity; Fig. 3), the use of anaerobic glycolysis, and the storage of lactate.

In addition to NKA, we also measured catalase activity, an enzyme that scavenges reactive oxygen species (ROS) by decomposing reactive hydrogen peroxide to water and oxygen (Felton 1995). Generation of ROS is known to be positively correlated with ambient oxygen concentrations, where hyperoxia conditions lead to increased ROS production (Rifkind et al. 1993; Felton 1995). There are a number of animals that face the challenge of abrupt oxygen fluctuations, and most have been shown to modify their antioxidant systems in preparation for re-oxygenation by either increasing antioxidant enzyme activity or avoiding oxidative stress by reducing metabolic rate (Lushchak et al. 2001; Storey 2007). Although we do not know the status of ROS generation in the gill of midshipman in this study, we do see a significant decrease in catalase activity (Fig. 3b), suggesting a depressed metabolic rate may assist in reducing ROS formation during hypoxia, although further investigation is necessary.

The remarkable ability of the plainfin midshipman to tolerate repeated bouts of aquatic hypoxia can be attributed to their ability to rapidly suppress metabolic rate at the initial onset of hypoxic conditions, reduce ATP consumption, and tolerate a buildup of anaerobic byproducts. By examining midshipman in their natural

environment, we have identified that Type-I males appear to have a greater tolerance of aquatic hypoxic exposure. This is likely related to the physical exertion of the Type-I males during parental care, as they remain on their nest for prolonged periods. It should be noted that this study only examined the effects of aquatic hypoxia, and does not account for other respiratory media. One possibility that is overlooked is the midshipman Type-I males breathing air as the tide recedes; we have observed several nest sites that were completely devoid of water. Survival of the midshipman in the intertidal zone may not only be attributed to tolerance of aquatic hypoxia and metabolic byproducts, but mitigated by air emergence and breathing, as demonstrated in this species by Martin (1993). Furthermore, as the midshipman eggs are retained in a thin film on the underside of rocks (Coleman 1999), they are likely exposed to air in the nest during low tide. A key selective pressure for intertidal breeding is the access to abundant oxygen and higher temperatures to accelerate growth and development (Martin and Strathman 1999). It is likely that the Type-I midshipman also takes advantage of the abundant oxygen present in the air chamber formed during low tide (personal observations). Currently, we are investigating the growth and development of the eggs under various aquatic oxygen tensions, with additional studies to compare the physiological traits aforementioned between the Type-I, -II males, and female morphs of the species to gain further evidence of the superior tolerance mechanisms of the Type-I males. The evolutionary strategies for teleost reproduction in the intertidal zone remain a fascinating topic.

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