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Nesting on high: reproductive and physiological consequences of breeding across an intertidal gradient

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Abstract

Nest site selection is a critical parental decision with profound fitness consequences, yet the physiological consequences of these decisions are rarely examined. Certain fishes and other aquatic organisms construct nests and provide parental care in the intertidal zone—an environment characterized by fluctuating water levels, which can exert intermittent and sometimes extreme abiotic stress on the animals that live there including dramatic changes in temperature and dissolved oxygen level. In this study, we used the plainfin midshipman fish, *Porichthys notatus*, to test whether (1) nest site preferences and reproductive success vary across an intertidal elevation gradient, and (2) fish that nest at higher elevations pay greater physiological costs due to prolonged exposure to more extreme abiotic conditions. We found that fish preferred nests lower in the intertidal zone, with larger males outcompeting smaller males for these sites. Broods at high elevations suffered greater offspring mortality than broods at lower elevations. The average microhabitat temperature of nests was also warmer and more variable at higher elevations compared to lower elevations. While isolated from the ocean during low tides, care-giving parents increased their use of anaerobic metabolism, and potentially draw upon oxygen reserves in the swim bladder. Our results suggest that the choice of nesting location can have profound effects on a parent's physiology and may generate significant variation in reproductive success among individuals.

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Introduction

The decision of where to construct a nest and raise young is one of the most critical choices a parent can make, and this decision can have strong fitness consequences (Resetarits 1996; Refsnider and Janzen 2010). Parents are expected to choose nesting sites according to the fitness benefits that those sites provide, weighed against the costs of acquiring or building nests, and caring for young at those sites. For example, some birds will nest in areas that are more exposed to the elements if those locations are also safer from predators (e.g. dusky warblers, *Phylloscopus fuscatus*, Forstmeier and Weiss 2004; piping plovers, *Charadrius melodus*, Mayer et al. 2009). In some reptiles, females that brood their young in cool sites experience less predation pressure, but this also lengthens offspring development time and increases the cost of parental care to females, who do not forage during the care period (e.g. pythons, *Liasis fuscus*, Madsen and Shine 1999). Animals are expected to prefer to breed in areas where their reproductive output will be optimized, where offspring survival is high and where the physiological costs of providing care are not prohibitive. However, the physiological costs associated with these decisions are rarely examined.

Some fishes and other aquatic organisms build their nests, deposit eggs, and care for offspring in semi-terrestrial or even fully terrestrial near-shore environments (Martin et al. 2004). Such beach- or intertidal-spawning has evolved multiple times across distantly-related fish clades and is thought to be favoured because breeding above the waterline provides the young with warmer incubation temperatures, increased oxygen availability, and safety from aquatic predators (Martin and Swiderski 2001). The enhanced oxygen diffusion and higher average temperatures that eggs experience in the intertidal zone, especially if exposed to air, can accelerate development (Bermudes and Ritar 1999; Smyder and Martin 2002), thereby benefitting parents by reducing the duration of care. However, routine immersion and emersion by the cycling tides may also present a considerable physiological challenge. Temperature extremes, desiccation risk, and irradiance all typically increase in intensity and/or duration with elevation in the intertidal zone (Raffaelli and Hawkins 1996; Yamahira 1997), and tolerance of these extremes can strongly dictate the vertical distribution limits of intertidal organisms (Somero 2002; Tomanek and Helmuth 2002). Furthermore, if eggs and parents are not exposed to air at low tides, but rather submerged in tidepools, they can experience reduced pH and extreme aquatic hypoxia (Truchot and Duhamel-Jouve 1980; Richards 2011). Thus, for an intertidally-breeding fish, the choice of how high or low in the intertidal zone to build a nest and care for eggs must involve weighing the fitness payoffs of reproducing there against the potentially severe physiological costs of exposure to abiotic stress. Nest site choice is therefore an optimization problem, in which the relative costs and benefits of a particular choice will depend on (1) the ability of the parents (and offspring) to physiologically cope with the abiotic stressors of the location, (2) the parents' residual reproductive value, and (3) the parents' ability to compete for and retain their optimal nest site.

Here, we used the plainfin midshipman fish, *Porichthys notatus*, to investigate how reproduction and physiology are affected by elevation in the intertidal zone. *P. notatus* is a species of marine toadfish that breeds in the rocky intertidal zones along the west coast of North America. At the onset of the breeding season in late April, large reproductive males (called guarder or Type I males) migrate from the deep ocean to the intertidal zone and compete for nests, which they construct by excavating cavities in the soft sediment beneath large intertidal rocks (Arora 1948). Males possess a large physoclistous swim bladder that has been co-opted for sound production by the drumming action of two specialized sonic

muscles (Bass and Marchaterre 1989). Guarder males acoustically court gravid females. Gravid females, swim freely through the submerged intertidal zone using phonotaxis to locate preferred males in their nests and then will lay eggs on the underside of a male's defended rock (i.e. the ceiling of the nest cavity, Brantley and Bass 1994). The males remain in their intertidal nests to provide sole paternal care for their eggs for a prolonged 3-month parental care period, over which time they regularly experience isolation from the ocean due to the tidal cycles (Arora 1948; Cogliati et al. 2013). Often, the nesting cavity retains a small pool of water at low tide that can quickly become hypoxic (Craig et al. 2014, Fig. 1a), though at other times, the nest cavity drains completely to leave both the eggs and the care-giving male exposed to air for multiple hours during the low tide (Fig. 1a). Thus, midshipman males that remain confined to their nests to care for offspring may sometimes experience dry nest conditions, and at other times experience submersion in small hypoxic pools during low tides. The physiological mechanisms by which plainfin midshipman cope with these changing conditions are not well understood. Earlier work has suggested that males could use a combination of metabolic depression, recruitment of anaerobic metabolism (Craig et al. 2014; LeMoine et al. 2014), and a strong capacity to regulate blood pH (Perry et al. 2010) when submerged in hypoxic waters. Following low tide hypoxia exposure, males are likely to have to pay physiological recovery costs in the form of lactate oxidation, gluconeogenesis, and re-establishment of acid–base homeostasis (Dunn and Hochachka 1986; Thomas et al. 1986; Richards 2009). If emergence from the hypoxic water is possible, then midshipman males may also do so to breath air via their skin and gills (Martin 1993), as seen in some other intertidal fishes (Martin 2014). It is possible that *P. notatus* also uses its swim bladder as an oxygen reservoir during periods of hypoxia or air exposure, as has been previously observed in the longjaw mudsucker (*Gillichthys mirabilis*) and in the European eel (*Anguilla anguilla*) (Berg and Steen 1965; Todd and Ebeling 1966), though no study has yet directly examined this idea. In this study, we used the plainfin midshipman to investigate the following interrelated questions: (1) Does elevation in the intertidal zone affect nest site preferences? (2) Is there an optimal nesting elevation that maximizes the reproductive payoff when breeding in the intertidal zone? (3) What physiological changes do parental care-giving males undergo when their nests are isolated from the ocean during low tide?

Methods

Study 1: How does reproductive success change across an intertidal elevation gradient?

On April 27 and 28, 2017, we constructed 51 artificial nests using square (12"×12") concrete tiles in the intertidal zone of a private beach located in Dabob Bay of the Hood Canal, Washington (47°76′N, 122°86′W). Plainfin midshipman males readily use such tiles as nests (DeMartini 1988, 1991; Bose et al. 2016). These tiles allowed us to hold the structure and size of the nests constant while varying their elevation in the intertidal zone. We placed the artificial nests in five rows of 10 nests per row (with one row having 11 nests) parallel to the waterline at differing elevations (−1.9, −1.5, −1.1, −0.4. and +0.2 ft relative to chart datum). In each row, nests were placed 2.5 m apart from one another. The lowest row of artificial nests was placed just above the lowest low water mark during the spring tide of April 2017 and all the other rows of nests were placed progressively higher
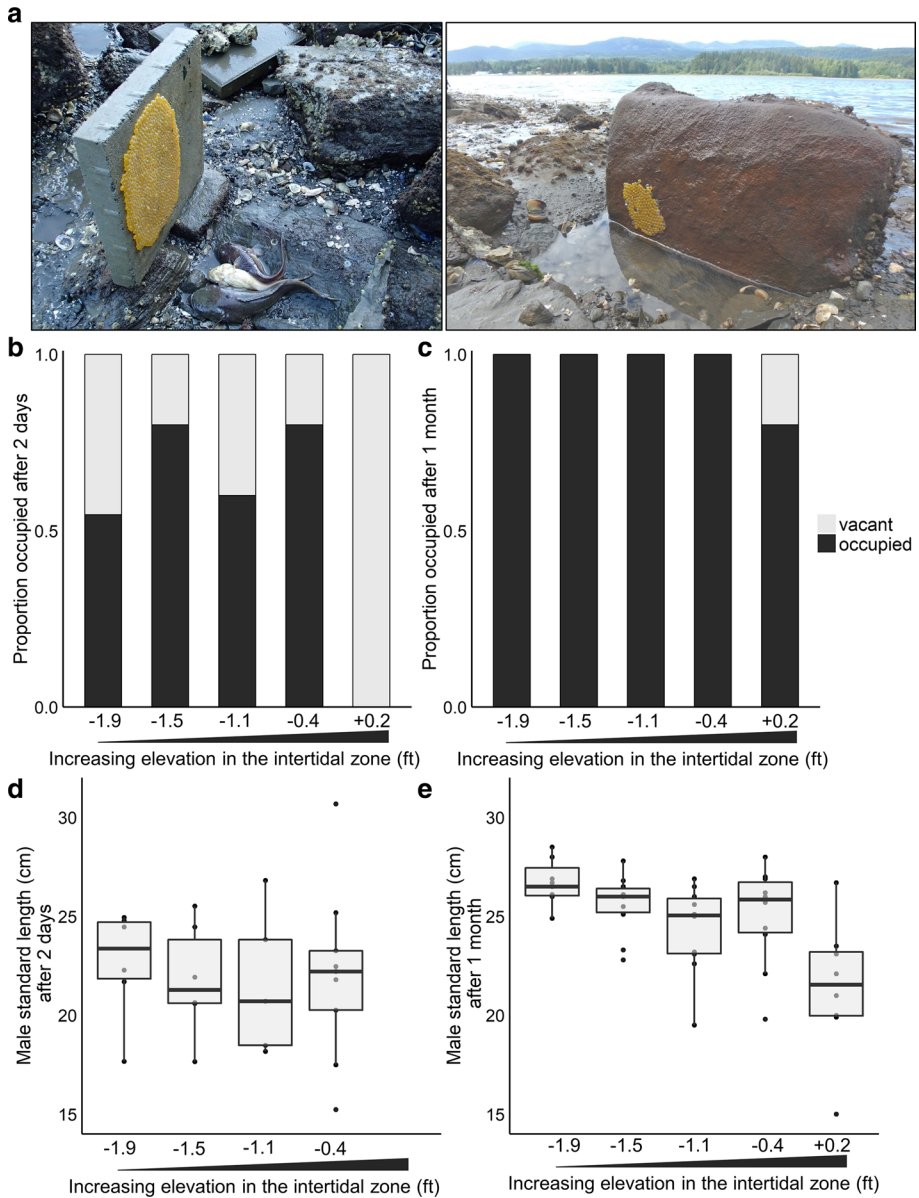
Fig. 1 Nest occupation and male size across an intertidal elevation gradient. **a** The left panel depicts a plainfin midshipman nest with eggs beneath a concrete tile, while the right panel depicts a nest with eggs beneath a naturally-occurring intertidal rock. Note the male and females in the nest on the left. The nest beneath the tile happened to drain of water, while the nest on the right happened to retain a pool of water. Proportion of nests occupied by males **b** 2 days and **c** 1 month after nest construction. Male body size across intertidal elevation gradient, **d** 2 days and **e** 1 month after nest construction

through the vertical elevation gradient where midshipman fish naturally nest. Temperature loggers (Thermochron iButtons, model DS1921G) were placed on the egg-laying surface in 23 of these nests across the elevation gradient and recorded hourly temperature measurements (to the nearest 0.5 °C). Note, the loggers did not affect nest occupation by males or the number of eggs acquired. Two days after constructing these nests, we recorded how many were occupied by a guarding male. All males were photographed next to a ruler and the male's standard length was measured from the photos using ImageJ (v1.48).

We then returned to these nests 1 month later on May 28, 2017. Plainfin midshipman males compete over nest ownership and nest take-over events are common, especially during the first month of the breeding season (DeMartini 1988; Cogliati et al. 2013; Bose et al. 2014). Thus, after 1 month, we expected that many of the original males would have been replaced by more competitive males, and that the distribution of male body size across the intertidal elevation gradient would reflect the value of nesting at those sites (analogous to an ideal despotic distribution, Fretwell 1972). Again, we recorded how many nests were occupied by males, retrieved the temperature loggers, photographed each male to measure standard length, and photographed any eggs present on the tile surface to quantify total brood size (see Fig. 1a). Total brood size was quantified by adding the number of live and dead eggs in each nest (see Fig. 2). Live eggs can easily be recognized based on their yellow or orange translucent colour. Dead eggs, on the other hand, could be determined in two ways. Dead eggs that are still attached to the nest surface appear white and opaque, while dead eggs that have since disintegrated and washed away leave behind distinctive visual markings from the adhesive discs that originally attached the eggs to the nest surface (henceforth called "egg scars"). Our sampling took place well before any offspring were developed enough to detach from the rock surface and become free swimming. Therefore, egg scars in this study are a reliable indicator of egg mortality.

Fig. 2 Proportion of eggs per nest surviving after 1 month across the intertidal elevation gradient
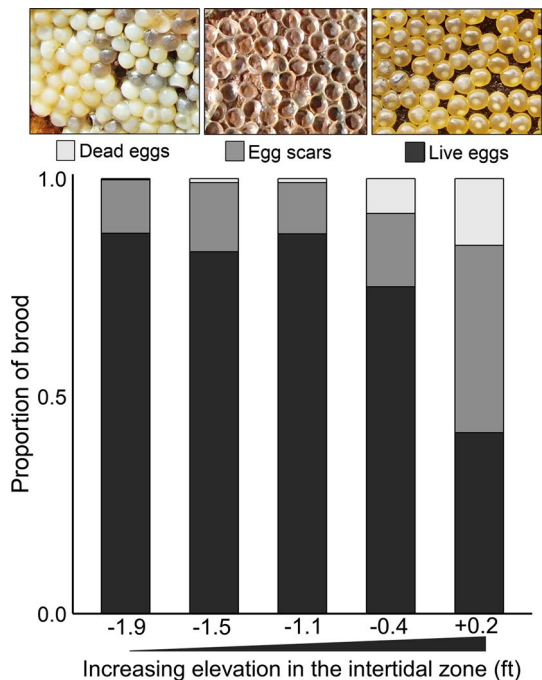
All analyses were performed in the statistical software R (version 3.3.1, R Development Core Team 2016). We tested whether a particular elevation (row of nests) in the intertidal zone was the first to be occupied or had a higher occupancy rate using a 5-sample Chi square test for equal proportions. We then used a linear model (LM) to test whether a particular elevation (or row) attracted larger males after 2 days, specifying male standard length as the response variable and intertidal elevation as the categorical predictor variable. We also tested whether the males that had occupied nests after 2 days were subsequently replaced by larger, more competitive males after 1 month. To do this, we used the nests that were occupied at both timepoints, took the standard lengths of both the male occupants and fit them with a paired t test. We also tested whether after 1 month, there was a particular elevation (or row) with higher male occupancy than the others, using another 5-sample Chi square test for equal proportions. We then fit two more models to test which rows held larger males (a LM) and larger broods (a generalized linear model, GLM, assuming a quasi-poisson error distribution, Kabacoff 2011), specifying intertidal elevation as the categorical predictor variable in both models, and male standard length as a covariate in the model for total brood size. Multicollinearity between male standard length and intertidal elevation was low (variance inflation factor (VIF) = 1.7, Zuur et al. 2010). We also tested whether the proportion of live eggs in each brood varied with intertidal elevation using a GLM assuming a quasi-binomial error distribution (Kabacoff 2011), and again included elevation as the categorical predictor and male standard length as a covariate (VIF = 1.6).

We calculated daily average temperatures, daily standard deviations in temperature, and daily high temperatures (measured as the 95% percentile for each set of daily temperature measurements) for each of the 23 nests with temperature loggers. We then calculated the average of each of these measures across the month for every nest and fit them with LMs to investigate how nest temperature varied with intertidal elevation.

Study 2: What physiological changes do *P. notatus* males undergo across the duration of a low tide?

Between May 22–26, 2017, we located 35 males in their nests during the ebbing tides in the intertidal zone at Crescent Beach, British Columbia (49°2′N, 122°53′W). Nests were located by gently overturning intertidal rocks to expose the nesting cavity. The nesting cavity often contained a small tidepool of water in which the male would be partially or completely immersed. For every nest we found we attempted to find a second nest at the same intertidal elevation that contained a similar-sized male. As the tide receded, we immediately sampled one of these two males as soon as their nest was uncovered (sampling typically occurred within 1 min or less of nest isolation from the ocean). This first male was quickly euthanized and dissected on-site, while we left the male in the second nest in place for the duration of the low tide cycle. We euthanized and dissected these second males several hours later, just before each nest would have been flushed with ocean water by the incoming tide. We measured water temperature (°C), pH (Oakton PCSTestr 35), dissolved oxygen (mg/L, Oakton RDO 450), salinity (ppt, refractometer), nitrate (NO_3^-, ppm), nitrite (NO_2^-, ppm), and ammonia (NH_3, ppm, with an API® Saltwater aquarium test kit) from the small tidepool of water retained in each nest when we removed each male. Thus, we sampled males who had just been exposed to either, (1) complete immersion in ocean water during the preceding high tide, or (2) isolation from the ocean in a nesting cavity, which typically retained a small amount of water, for the duration of the low tide.

Each removed male was sampled by placing them in a bath of benzocaine (dissolved in 95% ethanol, final working concentration 1 g L^{-1}) followed by quick cervical severance with a scalpel blade. We then measured them for standard length (to the nearest 0.1 cm) and total body mass (to the nearest 0.2 g). A blood sample (~0.5 mL) was taken from the caudal vein using a syringe. The whole blood was quickly transferred into a micro-centrifuge tube, spun for 1 min at 2000×g (VWR Galaxy MiniStar Microcentrifuge), and the plasma was isolated and snap frozen in liquid nitrogen. We also collected a sample of blood into a 2 μL microhaematocrit tube, which we spun for 10 min at 6900×g (LW Scientific ZipCombo Centrifuge) to determine haematocrit. The whole brain and samples of the liver and axial muscle (muscle taken from the dorsal section of the tail just posterior to the body cavity) were excised from the fish, weighed (to the nearest 0.01 g), quickly freeze-clamped and then transferred to liquid nitrogen. Our freeze-clamped tissues were consistently frozen within 60–90 s after euthanasia. All samples were stored at −80 °C until the tissue could be assayed.

We also removed the swim bladder, with both sonic muscles still attached, and measured its volume in 0.9% saline by standard volumetric methods (following Scherle 1970). After determining the swim bladder volume, we used a retractable needle-type fibre-optic oxygen sensor (Pyroscience FireStingO2) housed in a syringe needle to measure the partial pressure of oxygen within the swim bladder. The swim bladder was first penetrated with the needle and then the probe was carefully advanced out of the needle and into the swim bladder lumen, where the partial pressure of oxygen was recorded.

Frozen muscle, liver, and brain tissue samples from 33 of the 35 sampled males were ground into a fine powder using a mortar and pestle that were pre-cooled with liquid nitrogen. We analyzed each tissue sample for lactate, glucose, and glycogen content via standard spectrophotometric methods, adapted for a 96-well plate format (Bergmeyer 1983). Briefly, a known mass of powdered tissue (~40 to 60 mg) was homogenized for 20 s in 300 μL of ice-cold 6% HClO$_4$ using the highest setting of a PowerGen 125 electric homogenizer (Fisher Scientific, Whitby, ON, Canada). Homogenates were vortexed, and 100 μL of the acidified extract was immediately frozen in liquid nitrogen for later analysis of glycogen and glucose content. The remaining homogenate was centrifuged at 4 °C for 10 min at 10,000 g. The supernatant was transferred to a new microcentrifuge tube, neutralized (6.8 ≤ pH ≤ 7.2), and then centrifuged again at 10,000g for 10 min at 4 °C. The resulting supernatant was immediately used for quantification of lactate content by measuring the change in absorbance at 340 nm under the following assay conditions: 2.5 mM NAD$^+$ and excess coupling enzyme (lactate dehydrogenase) in buffer containing 0.6 M glycine and 0.5 M hydrazine sulphate (pH = 9.4). Glycogen and glucose content were determined by adding 50 μL of 1 M K$_2$HCO$_3$ and 100 μL of 400 mM acetate buffer (pH = 4.8) to the 100 μL of acidified homogenate. This solution was briefly vortexed and split into two 125 μL aliquots: one was digested for 2 h at 40 °C by adding 7 μL of amyloglucosidase (~30 U total per aliquot, suspended in a buffer containing 300 mM Tris–HCl and 4.05 mM MgSO$_4$ at pH = 7.5), which breaks down glycogen into single glucose units by catalyzing the hydrolysis of terminal glucose residues, and the other aliquot was not digested and simply incubated at 4 °C for 2 h. Each aliquot was neutralized following incubation. Undigested and digested samples were assayed for glucose content under the following assay conditions: 1 mM ATP, 0.5 mM NADP$^+$, 5 mM MgCl$_2$, and excess coupling enzymes (hexokinase and glucose-6-phosphate dehydrogenase), in 20 mM imidazole (pH = 7.4). The glucose measurement in the undigested aliquot represented the free glucose content of the sample, while the difference in glucose content between the digested and undigested aliquots represented the glycogen content of the sample. All metabolite assays were run in

duplicate at 37 °C on a SpectraMax Plus 384 microplate reader with temperature control (Molecular Devices, Sunnyvale, CA, USA). Tissue protein content was also quantified following Bradford (1976). Powdered tissue was dried in an oven at 60 °C for 48 h to determine the dry mass for a given wet mass of powdered tissue. This allowed us to express tissue metabolite contents relative to dry tissue mass. We also determined relative tissue water content from the difference between wet and dry tissue masses for the same sample, as described by Lisser et al. (2016).

To reduce the number of statistical tests employed, we used a principal component analysis (PCA) to evaluate the variation in tissue metabolite concentrations (lactate, glycogen and glucose in the liver, brain, and muscle). All variables were log-transformed to improve the symmetry of their distributions and then standardized by mean-centering and dividing by their standard deviations before the PCA. The first principal component (PC1) captured the greatest percentage of variation in metabolite concentrations (summarized in Table 1). Glycogen typically had the opposite effect on PC1 than lactate (i.e. an end product of anaerobic glycolysis) and glucose (a breakdown product of glycogen). Thus, increases in PC1 indicate a depletion of high-energy compounds and/or an increased use of anaerobic metabolism, and we therefore used PC1 as a composite variable that provides an index of tissue metabolic disturbance. Supplementary Materials Table 1 summarizes the additional metabolites that we measured but did not include in the PCA (plasma lactate as well as protein and water contents from liver, muscle and brain).

We tested whether the degree of physiological disturbance, as reflected by PC1, increased with the time spent isolated from the ocean during the low tide. To test this, we fit a LM, specifying PC1 as the response variable, time spent isolated (in h) as a continuous predictor variable as well as male standard length and date of sampling as covariables. A Yeo-Johnson power transformation was applied to PC1 to improve normality and homoscedasticity of the model's residuals. We next tested whether haematocrit and plasma lactate (after log-transformation) increased with time spent isolated (in h), by fitting it with

Table 1 Summary of tissue metabolite contents (μmol g^{-1} tissue dry wt) from male *P. notatus* sampled either immediately after, or several hours after, isolation from the ocean due to the low tide

Metabolites	Males sampled immediately upon isolation from ocean		Males sampled several hours after isolation from ocean		Principal component 1 loadings
	Mean ± SD	Minimum–maximum	Mean ± SD	Minimum–maximum	
Liver glycogen	154.8 ± 49.8	103.6–307.2	148.5 ± 44.3	61.9–225.2	− 0.23
Liver lactate	2.6 ± 0.8	1.2–3.8	3.8 ± 1.5	1.7–7.3	0.51
Liver glucose	15.1 ± 7.2	5.5–27.7	29.3 ± 17.9	10.1–62.2	0.40
Brain glycogen	33.4 ± 8.8	21.5–55.9	26.9 ± 16.3	10.1–65.6	− 0.31
Brain lactate	17.8 ± 5.6	9.0–27.8	29.6 ± 11.0	14.1–45.2	0.51
Brain glucose	5.8 ± 2.5	2.5–11.2	9.1 ± 4.4	4.1–18.4	0.28
Muscle glycogen	19.1 ± 18.1	4.3–79.3	24.1 ± 19.3	8.0–80.4	0.10
Muscle lactate	31.5 ± 10.3	10.2–49.8	41.6 ± 21.8	16.6–104.2	0.36
Muscle glucose	3.2 ± 1.1	1.8–5.9	3.3 ± 1.9	0.9–7.0	0.22
Variation explained					27.4%

These metabolite contents from the 33 analyzed males were reduced into a single composite variable using a principal component analysis

a LM that included the same independent variables as described above (the exponential of time spent isolated was included in the model of plasma lactate).

Finally, we tested whether the time spent isolated from the ocean affected the amount of oxygen stored within the swim bladder. We used the measurements of oxygen partial pressure and volume for each bladder to calculate the volume of oxygen (in mL) that was held within each bladder. We fit the volume of oxygen in each swim bladder (mL, log-transformed) as our response variable in a generalized least squares model (GLS) with a power variance function (using 'varPower' in the 'nlme' package, Pinheiro et al. 2016) to accommodate the heterogenous (increasing) residual variance that we observed as males spent more time in nests isolated from the ocean. We included male body mass (g, log-transformed) as a covariate as well as the exponential of time spent isolated (in h) as our predictor variable. We also included date of sampling as a covariable in the model.

Ethical note

The plainfin midshipman fish is a common intertidal species and not considered threatened or endangered (Collette et al. 2010). A Department of Fisheries and Oceans Canada scientific collections permit was obtained for all animals that were terminally sampled in this study (permit no. XR 58 2017). The procedures used in these studies were approved by the McMaster University Animal Research Ethics Board (AUP number 13-12-52) and are in line with the guidelines set by the Canadian Council on Animal Care (CCAC).

Results

Study 1: Reproductive success declines with increasing intertidal elevation

Two days after laying out the artificial nests on the beach, all but those at the highest elevation were occupied by nesting males ($\chi^2 = 20$, $df = 4$, $P = 0.002$, Fig. 1b). At this early sampling time point, the body lengths of the males did not differ across the intertidal elevations (LM, $F_{3,22} = 0.09$, $P = 0.97$, Fig. 1d). After 1 month, virtually all of the nests across the elevation rows were occupied (Fig. 1c, only two out of the 51 total nests, both at the highest elevation, were still unoccupied). The males at the highest intertidal elevation were smaller than males at the lower elevations ($F_{4,44} = 7.15$, $P = 0.00016$, Fig. 1e) while nesting males at the other elevation rows did not differ in body size (all $P > 0.13$). However, the males found occupying nests after 1 month were on average larger than the original males 1 month prior (paired t test, $t_{25} = 5.15$, $P < 0.0001$).

Broods contained an average ($\pm$ SD) of 877 ± 478 live eggs. Total brood size (live eggs + dead eggs + scars) tended to increase with male size (GLM, est. $\pm$ SE $= 0.055 \pm 0.028$, $t_{42} = 2.00$, $P = 0.052$) but did not differ across the elevation gradient (all $P > 0.14$). A smaller fraction of the broods survived in the highest elevation row compared to the other four lower elevations (GLM, contrast made between the highest row and the average of all the lower rows: est. $\pm$ SE $= 1.60 \pm 0.45$, $t_{42} = 3.59$, $P = 0.0003$, Fig. 2). The proportion of the brood surviving did not differ among the other intertidal elevations (all $P > 0.17$). Male standard length did not correlate with the proportion of brood surviving (LM, est. $\pm$ SE $= 0.067 \pm 0.070$, $t_{42} = 0.96$, $P = 0.34$).

Across our 1-month study, the fish and eggs in the artificial nests placed along the intertidal gradient experienced temperatures ranging from 9.5 to 30.5 °C. The maximum
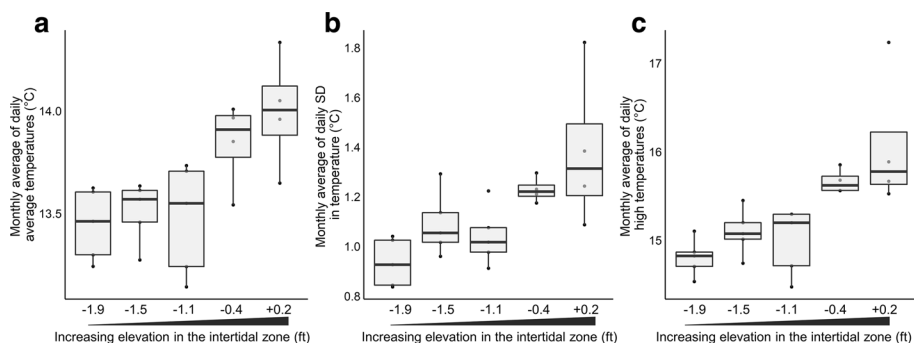
Fig. 3 Monthly averages for nest temperature measured across the intertidal elevation gradient: **a** daily average temperatures, **b** daily standard deviations in temperature, and **c** daily high temperatures. Data loggers were placed on the rock surface (ceiling of each nesting cavity) and therefore recorded temperature of the environment where the eggs where laid

Table 2 Summary of abiotic parameters measured from the small pools of water retained in plainfin midshipman nests following their isolation from the ocean during low tide

	Tide pools sampled immediately upon isolation from ocean		Tide pools sampled several hours after isolation from ocean	
	Mean ± SD	Minimum–maximum	Mean ± SD	Minimum–maximum
Time spent isolated (h)	0 (17)	–	3.83 ± 1.63 (18)	1.25–6.82
Water temperature (°C)*	16.3 ± 2.2 (17)	13.2–20.2	20.4 ± 3.8 (14)	15.8–26.5
Dissolved oxygen (mg/L)*	6.03 ± 3.38 (17)	1.7–15.5	3.37 ± 2.15 (14)	0.5–6.92
pH	8.0 ± 0.3 (17)	7.6–8.7	8.0 ± 0.4 (14)	7.4–8.5
Salinity (ppt)*	22.5 ± 3.7 (17)	16–30	14.9 ± 5.2 (14)	8–25
Ammonia (ppm)	0.16 ± 0.28 (17)	0–1	0.21 ± 0.29 (13)	0–1
Nitrite (ppm)	0.16 ± 0.49 (17)	0–2	1.17 ± 2.99 (13)	0–10
Nitrate (ppm)	6.24 ± 19.3 (17)	0–80	4.65 ± 11.1 (13)	0–40

Sample sizes are reported in parentheses

*Significant difference ($P < 0.05$) between groups, as tested by a Welch two-sample t test

temperature recorded at each of the five elevations/rows of tiles from lowest to highest in the intertidal zone was 26.5, 28.5, 28.5, 27.5, and 30.5 °C. Nests higher in the intertidal zone experienced not only higher daily *average* temperatures (LM, $F_{4,18} = 5.58$, $P = 0.0042$, Fig. 3a), but also higher daily *standard deviations* in temperature ($F_{4,18} = 5.14$, $P = 0.0061$, Fig. 3b), and higher daily *high* temperatures ($F_{4,18} = 7.45$, $P = 0.001$, Fig. 3c).

Study 2: Nesting males suffer metabolic disturbance during the low tide

Abiotic conditions of the nest cavities became more challenging with prolonged isolation from the ocean during the low tide; nest cavity temperatures increased while the dissolved oxygen and salinity measures in the nest decreased (Table 2). Males that spent more time
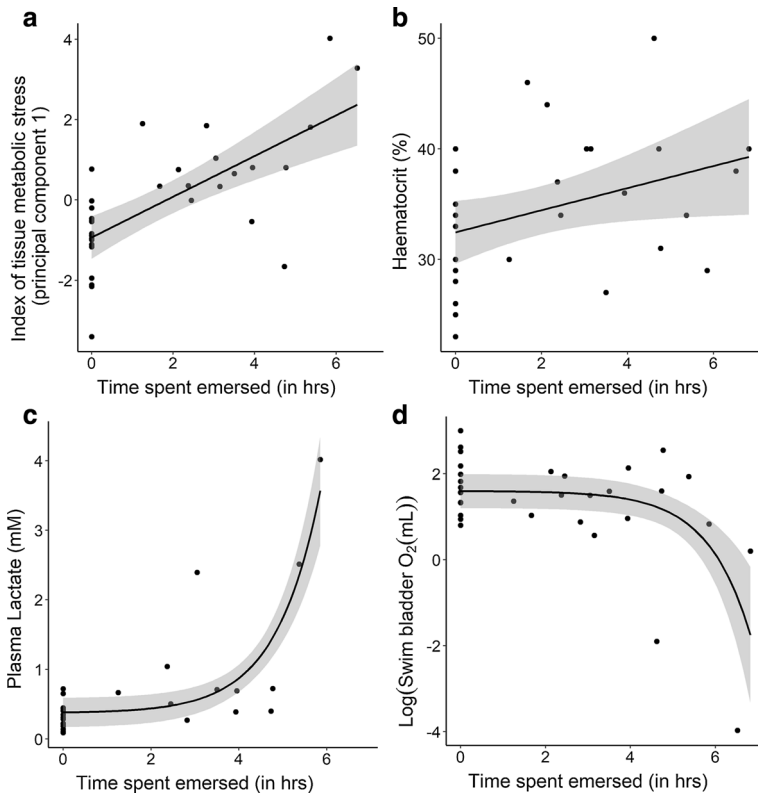
Fig. 4 Physiological changes across the duration of a low tide cycle as captured by **a** an index of tissue metabolic disturbance (PC1 in a principal component analysis of metabolite levels in brain, liver, and muscle; see "Methods" for details), **b** haematocrit (%), **c** plasma lactate (mM), and **d** swim bladder O_2 stores. Note that panel A shows the Yeo–Johnson transformed PC1 values, while the other plots show untransformed or log-transformed data with ordinary least squares or exponential regression fits

isolated from the ocean exhibited higher tissue metabolic disturbance, as captured by PC1 (LM, est. $\pm$ SE $= 0.48 \pm 0.09$, t $= 5.34$, $df = 28$, $P < 0.0001$, Fig. 4a). PC1 was associated with increased lactate and glucose levels across tissues, and decreased glycogen levels in the brain and liver. Plasma haematocrit increased with time spent isolated (LM, est. $\pm$ SE $= 1.11 \pm 0.48$, t $= 2.32$, $df = 28$, $P = 0.028$, Fig. 4b). Tissue water content did not change with time spent isolated from the ocean (Suppl. Materials Table 1). Males that spent more time isolated also exhibited exponentially increasing lactate concentrations in their plasma (LM, est. $\pm$ SE $= 0.0034 \pm 0.00076$, t $= 4.44$, $df = 22$, $P = 0.0002$, Fig. 4c, Suppl. Materials Table 1). Swim bladder oxygen decreased exponentially with increasing time spent isolated from the ocean (GLS, est. $\pm$ SE $= -0.004 \pm 0.0012$, t $= -3.43$, $df = 28$, $P = 0.0019$, Fig. 4d).

Discussion

We investigated how reproduction and physiological disturbance in a teleost fish are affected by nest elevation in the intertidal zone. Males nesting *higher* in the intertidal zone experienced *longer* periods of isolation from the ocean and greater abiotic extremes during low tides than males nesting at lower elevations. High elevation males consequently experienced greater physiological disturbances during low tides and also suffered reproductive costs.

High intertidal nesting sites are not preferred and yield low reproductive success

Although *all* elevations in the intertidal zone were eventually settled, nests lower in the intertidal were the first to become occupied. It is possible that this bottom-up pattern of residency occurs if males simply occupy the first vacant nesting site that they encounter when swimming into the intertidal zone from depth. However, midshipman males also compete intensely for nest ownership resulting in males occupying nests in a size-assortative manner, with the largest, most competitive males occupying the highest value nesting sites (DeMartini 1988, 1991; Bose et al. 2014, 2018). Over time, lower elevations in the intertidal became inhabited by larger males than the highest elevations, despite the fact that nest size—a major determinant of nest quality (Bose et al. 2018)—was kept constant in our experiment. Only the highest elevation nests were inhabited by smaller males, suggesting that low nests experience more intense competition and could only be retained by the most competitive (i.e. largest) males.

Nests positioned at the highest intertidal elevation suffered greater offspring mortality compared to nests lower in the intertidal zone. Although broods at this elevation were also under the care of smaller and perhaps lower quality males, we did not find that male body size correlated significantly with offspring survival, suggesting that other factors (such as abiotic conditions) may drive offspring mortality. Abiotic factors, such as temperature and desiccation risk, increase in severity with intertidal elevation (Raffaelli and Hawkins 1996) and these factors can affect reproductive outcomes if they become too extreme (Martin and Strathmann 1999; Martin et al. 2004). Indeed, even within the shelters of the nesting cavities where the eggs are cared for (and are protected from direct sunlight), our temperature loggers recorded higher and more variable temperatures in the higher elevation nests (Fig. 3). Broods at the highest intertidal elevations were subjected to more extreme temperatures than broods at lower elevations. During low tides, broods would also have been exposed to more prolonged hypoxia if the nesting cavities retained water or more prolonged desiccation risk if the nesting cavities were completely drained of water. Offspring in high-elevation nests may also be more susceptible to terrestrial predators such as shore crabs (e.g. *Hemigrapsus* spp.), which are ubiquitous at many *P. notatus* intertidal breeding grounds (personal observations A.B., S.B.). Future studies that record parental care by males at various elevations could shed further light on the degree to which young survival is influenced by abiotic conditions in the nests.

Feeding opportunities for young may be greater in nests lower down in the intertidal zone. Midshipman eggs hatch approximately halfway through the developmental period that they spend within their nests. After hatching, the young remain attached to the rock surface via their yolk sac for approximately another 30 days before becoming independent. Since hatched young also feed on plankton within their nests (Crane 1981) higher elevation

nests may provide fewer foraging opportunities for offspring owing to the shorter cumulative times that they spend being submerged. In addition to potentially more foraging opportunities for the young, lower intertidal elevations could also experience less predation by terrestrial predators.

Smaller, less competitive males appear to be ousted to higher fringe locations of the intertidal zone where the physiological costs of reproduction are higher and/or reproductive success is lower. If small individuals are less capable of coping with abiotic stress compared with large individuals, as is observed in some other species (reviewed in Nilsson and Östlund-Nilsson 2008), then this could further explain why reproductive success at these higher locations was compromised. Specifically, for hypoxia resistance, smaller fish generally have greater mass-specific O_2 demands (Nilsson and Östlund-Nilsson 2008), and many physiological traits that contribute to hypoxia tolerance, such as the activity of metabolic enzymes and glycogen stores, are influenced by body mass (Davies and Moyes 2007; Vornanen et al. 2011) such that larger animals may have an advantage over smaller animals. Smaller individuals may therefore not cope with hypoxia (or temperature) as well as larger individuals and therefore lack the energy reserves to effectively reproduce or guard their nests. These high elevation nesting sites are likely near the upper boundary of where plainfin midshipman can profitably breed in the intertidal zone.

Breeding at high elevations incurs physiological costs

Midshipman males likely employ multiple physiological coping mechanisms to survive the challenging abiotic stressors in the intertidal environment. Males increased their use of anaerobic glycolysis at low tide, as indicated by increased lactate levels in the plasma, muscle, liver, and brain (Fig. 4, Table 1, Suppl. Materials Table 1). The increases in plasma lactate observed were comparable (although lower in magnitude) to those measured after 4-h hypoxia exposure (at an O_2 level of 15% of air-saturated water) in controlled lab conditions (Craig et al. 2014). Males may offset this recruitment of anaerobic metabolism somewhat by drawing upon oxygen reserves in the swim bladder, as observed in some other species during aquatic hypoxia or air exposure (*Anguilla anguilla*, Berg and Steen 1965; *Gillichthys mirabilis*, Todd and Ebeling 1966). Midshipman may also be capable of metabolic depression, which coupled with their low metabolic rate (~ 2.0 mmol kg^{-1} h^{-1}) compared to many other fishes (Craig et al. 2014; LeMoine et al. 2014) could also help them cope with O_2 limitation during low tides. Midshipman fish may even have some capacity for aerial exchange of O_2 and CO_2 via their skin and gills (Martin 1993; Martin et al. 2004). In addition to tracking physiological repercussions, future work should also aim to assess the behavioural responses of midshipman males to hypoxia as emergence from hypoxic tidepools is commonly seen in other intertidal fishes (Sayer and Davenport 1991; Martin 2014). Overall, these mechanisms may be particularly important during the protracted, 3-month breeding season of *P. notatus*, when males are routinely isolated from the ocean in their nests and have little capacity to forage and replenish energy stores (Bose et al. 2015; Cogliati et al. 2015).

There appeared to be greater physiological costs for males guarding nests and caring for offspring higher in the intertidal zone, and this may contribute to why males preferred nests at lower elevations (Fig. 1). Males are likely to pay physiological recovery costs following hypoxia exposure in the form of energetically expensive processes such as lactate oxidation, gluconeogenesis, and re-establishment of acid–base homeostasis. How such physiological costs directly impact reproductive output and adult survival will require

future investigation, but it is foreseeable that the metabolic costs of recovering from low-tide events could reduce the aerobic scope and tissue energy stores available to support subsequent behaviours such as brood tending, nest guarding, and courtship, and even influence future parental survival. For males nesting at high elevations, the repeated exposure to more extreme conditions during low tides may limit the ability of males to care for young. This may at least partially explain why smaller males that may be forced to breed at higher elevations in the intertidal zone achieve lower reproductive success. It should be noted that the weather on the particular days when we conducted our time series of dissections (May 22–26, 2017) was not unusually hot, cold, wet, or dry. Thus, the physiological patterns that we report here are likely representative of the physiological changes that fish would experience during a typical low tide event.

In summary, we have shown that nesting at high elevations in the intertidal zone hampers reproductive success and increases the physiological costs of breeding. High elevation broods experienced heavy mortality, which may be due to (1) abiotic extremes directly killing offspring, (2) increased susceptibility to terrestrial predators, and/or (3) physiological costs to parental males that may limit their ability to provide adequate parental care. Additionally, reduced foraging opportunities for offspring may also contribute to increased mortality in high elevation nests, though in our 1-month study none of the offspring we observed were yet old enough to feed exogenously. Future research will aim to disentangle the relative contributions of all these factors in driving offspring mortality across the intertidal zone. Overall, our results suggest that physiological limits in the ability to cope with harsh abiotic conditions may heavily influence the preference of beach-spawning fishes for particular nesting sites and elevations in the dynamic environment of the intertidal zone.

Author contributions AB, SB, BB, and GRS conceived and designed the study. AB and SB conducted the field work. BB conducted the laboratory assays. AB analyzed the data. AB and BB wrote the paper with input from all co-authors.

Compliance with ethical standards

Conflict of interest No competing interests declared.

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