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RESEARCH ARTICLE

Hyperoxia increases maximum oxygen consumption and aerobic scope of intertidal fish facing acutely high temperatures

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ABSTRACT

Daytime low tides that lead to high-temperature events in stranded rock pools often co-occur with algae-mediated hyperoxia as a result of strong solar radiation. Recent evidence shows aerobic metabolic scope (MS) can be expanded under hyperoxia in fish but so far this possibility has not been examined in intertidal species despite being an ecologically relevant scenario. Furthermore, it is unknown whether hyperoxia increases the upper thermal tolerance limits of intertidal fish and, therefore, their ability to withstand extreme high-temperature events. Therefore, we measured the metabolic response (mass-specific rate of oxygen consumption, $\dot{M}_{O_2}$) to thermal ramping (21–29°C) and the upper thermal tolerance limit (U_{TL}) of two intertidal triplefin fishes (*Bellapiscis medius* and *Forsterygion lapillum*) under hyperoxia and normoxia. Hyperoxia increased maximal oxygen consumption ($\dot{M}_{O_2,max}$) and MS of each species at ambient temperature (21°C) but also after thermal ramping to elevated temperatures such as those observed in rock pools (29°C). While hyperoxia did not provide a biologically meaningful increase in upper thermal tolerance of either species (>31°C under all conditions), the observed expansion of MS at 29°C under hyperoxia could potentially benefit the aerobic performance, and hence the growth and feeding potential, etc., of intertidal fish at non-critical temperatures. That hyperoxia does not increase upper thermal tolerance in a meaningful way is cause for concern as climate change is expected to drive more extreme rock pool temperatures in the future and this could present a major challenge for these species.

KEY WORDS: Oxygen consumption, Respirometry, Thermal tolerance, Thermal ramping, Metabolism

INTRODUCTION

Temperature has pervasive impacts on biological processes and plays an important role in shaping the distribution and abundance of species (Schulte, 2015). All fish face at least some natural fluctuation in environmental temperature, but species inhabiting intertidal rock pools can be exposed to large acute (hours) increases in temperature when daytime low tide coincides with hot terrestrial conditions (Fig. 1A). Low tide thermal ramping events in rock pools may ultimately threaten the upper thermal tolerance limits of intertidal fish, but are also associated with sub-lethal impacts including increased energetic demands. These higher energetic demands are reflected in the profound influence of temperature on aerobic metabolism where rates of oxygen consumption typically increase exponentially under acute thermal ramping (e.g. Healy and Schulte, 2012b; McArley et al., 2017). The aerobic metabolic response to thermal ramping has been well characterised in fish but there is a lack of studies investigating the role of aerobic metabolism in thermal tolerance in intertidal fish species, which are routinely exposed to acute high temperatures.

Thermal tolerance has been proposed to be linked to aerobic metabolism in aquatic ectotherms through the influence of temperature on aerobic metabolic scope (MS) (Portner and Farrell, 2008; Portner et al., 2017; Schulte, 2015; Sokolova et al., 2012). MS is the difference between basal (termed the standard metabolic rate, SMR) and maximum ($\dot{M}_{O_2,max}$) rates of oxygen consumption and theoretically represents the portion of metabolic capacity above and beyond basal requirements (i.e. SMR) that can be directed to aerobic-dependent activities such as growth, feeding, digestion, locomotion and reproduction (Claireaux and Lefrancois, 2007; Clark et al., 2013). As thermal ramping proceeds in rock pools, the MS of intertidal fish is expected to fall because higher temperatures increase SMR (Schulte, 2015), and the capacity to support aerobically driven activities may then decline at elevated temperatures. However, for MS to decline under thermal ramping, $\dot{M}_{O_2,max}$ has to increase less than SMR across an equivalent temperature increment. In some fish species, $\dot{M}_{O_2,max}$ may increase more than or in parallel with SMR at higher temperatures, and therefore MS fails to decline because $\dot{M}_{O_2,max}$ does not plateau (Healy and Schulte, 2012b; Lefevre, 2016; Norin et al., 2014). In the common triplefin (*Forsterygion lapillum* Hardy 1989), a species known to inhabit rock pools, $\dot{M}_{O_2,max}$ did not increase at higher temperatures following acclimation to 15, 18, 21 and 24°C (McArley et al., 2017). Furthermore, in the same study, routine $\dot{M}_{O_2}$ under acute thermal ramping to critical thermal limits (>29°C) only marginally exceeded $\dot{M}_{O_2,max}$ measured at the lower acclimation temperatures. Therefore, at least for this triplefin species, there may be a limited capacity to increase $\dot{M}_{O_2,max}$ at higher temperatures, and this would result in vulnerability to a collapse of MS when exposed to thermal ramping in rock pools.

Although the MS of at least one triplefin species appears constrained at higher temperatures (McArley et al., 2017), these findings were determined under normoxia (100% of air saturation) and this is not always the setting within rock pools. Thermal ramping in rock pools generally occurs on hot sunny days when rates of algal photosynthesis are high and rock pool water can become hyperoxic at this time (see Fig. 1B for example and Berschick et al., 1987; Bridges, 1988; Richards, 2011; Truchot and Duhamel-Jouve, 1980). Thus, in a thermal ramping event, intertidal fish reside within normoxic water at ambient temperatures as the tide first recedes; then, rock pool seawater warms and becomes increasingly hyperoxic until the incoming tide flushes the rock pool. $\dot{M}_{O_2,max}$ and MS were approximately doubled under hyperoxia compared with values under normoxia in European perch

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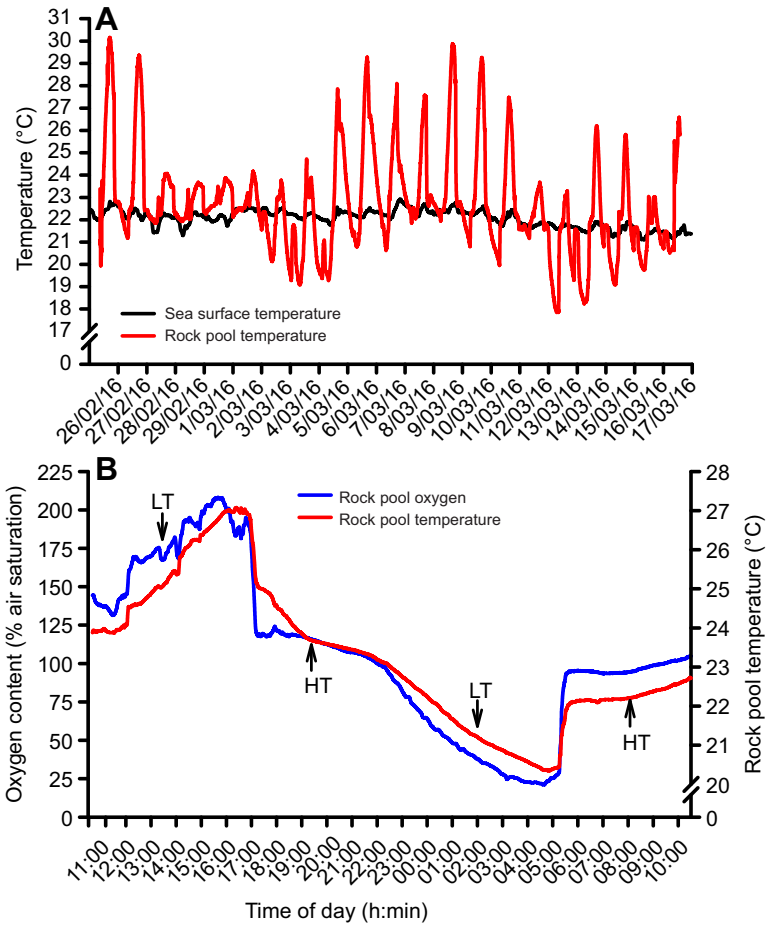

Fig. 1. Rock pool temperature and dissolved oxygen content. (A) The temperature of a rock pool known to be inhabited by intertidal triplefin species (red line) over a 3 week period during summer (February–March) 2016. The rock pool was located in the intertidal zone adjacent to Leigh Marine Laboratory in north eastern New Zealand. The black line shows sea surface temperature over the same 3 week period from coastal waters adjacent to the Leigh Marine Laboratory. (B) Water oxygen content (blue line, left axis) and water temperature (red line, right axis) of a rock pool known to be inhabited by intertidal triplefins over a 24 h period during summer. Upward facing arrows show the time of high tide (HT) and downward facing arrows show the time of low tide (LT).

(*Perca fluviatilis*) when measured at an acclimation temperature of 23°C (Brijs et al., 2015). Furthermore, in the same study, fish exposed to hyperoxia had a greater capacity to increase routine $\dot{M}_{O_2}$ at temperatures approaching upper tolerance limits (>30°C). It is not known whether the effects of hyperoxia seen in perch are widespread in fish but, if present in triplefins, an expected loss of MS under thermal ramping due to constrained $\dot{M}_{O_2,max}$ may be compensated for through the permissive effects of hyperoxia. MS could also be protected at high temperature under hyperoxia because the energetic costs of meeting oxygen demands (e.g. ventilatory costs) are diminished by excess oxygen availability (Mark et al., 2002; Portner et al., 2017). While environmental hyperoxia in rock pools may therefore offer a metabolic refuge for intertidal fish facing acute thermal stress, this possibility has not been addressed.

Intertidal organisms are assumed to live very close to their thermal tolerance limits (Helmuth et al., 2002). In rock pools inhabited by triplefin fish, we have measured temperatures exceeding 29°C in summer (Fig. 1A). These temperatures are close to the upper thermal tolerance limit (U_{TL}, 31.4°C) of *F. lapillum* (McArley et al., 2017), suggesting that, in at least some rock pools, this species would face temperatures close to its tolerable limit. While the exclusively intertidal triplefin [*Bellapiscis medius* (Günther 1861)] also appears to live close to its thermal limits (Hilton et al., 2008), the upper thermal limit of this species is unknown. An inability to match oxygen supply and demand has been proposed as a key determinant of thermal tolerance in aquatic ectotherms (Portner and Farrell, 2008; Portner, 2010; Portner et al., 2017) and experimental manipulations of tissue oxygen supply using hyperoxia have addressed the upper thermal limits of fish in this context (Brijs et al., 2015; Devor et al., 2016; Ekström et al., 2016; Healy and Schulte, 2012a; Mark et al., 2002). While these studies indicate that factors other than tissue oxygen supply may also set absolute upper thermal limits, hyperoxia did slightly increase the critical thermal maximum (+0.9°C) of European perch (Ekström et al., 2016). Rock pools are expected to become hyperoxic during thermal ramping events that should approach upper thermal limits, and for this reason, the possibility of increased maximal thermal tolerance under hyperoxia in intertidal fish requires consideration. Given predicted increases in the severity of heat wave events due to climate change (Perkins et al., 2012), and the possibility that intertidal fish already live close to their upper thermal limits, resolving thermal tolerance limits of intertidal fish under environmentally relevant conditions is pertinent.

The aims of this study were to determine: (1) whether hyperoxia increases the $\dot{M}_{O_2,max}$ and MS of intertidal fish during thermal ramping, (2) whether hyperoxia decreases routine $\dot{M}_{O_2}$ (an estimate of basal metabolic costs during rapidly changing temperature) in intertidal fish under thermal ramping, and (3) whether hyperoxia increases the upper thermal limits of intertidal fish. To address these aims, the $\dot{M}_{O_2}$ (mass-specific oxygen consumption) and upper thermal tolerance capacities were determined for two New Zealand triplefin fishes under hyperoxia (~200% air saturation) and normoxia (~100% air saturation). This included an exclusively intertidal species (*B. medius*) and a second species (*F. lapillum*), which also occupies intertidal rock pools but is more commonly found in shallow subtidal habitats. To address the first and second aims, MS was measured at 21°C, routine $\dot{M}_{O_2}$ was measured at 21, 24, 26, 28 and 29°C under thermal ramping and MS above routine

Journal of Experimental Biology (2018) 221, jeb189993. doi:10.1242/jeb.189993

metabolism was measured at 29°C (see Fig. 2). It was predicted that $\dot{M}_{O_2,max}$, and therefore MS, would be higher under hyperoxia at 21 and 29°C because of a limitation of $\dot{M}_{O_2,max}$ under normoxia (Brijs et al., 2015). Additionally, routine $\dot{M}_{O_2}$ under thermal ramping was predicted to increase less under hyperoxia than under normoxia because of decreased cardiorespiratory costs required to meet oxygen demands. As such, the rate and amplitude of gill ventilation were also measured under thermal ramping to determine whether the lower metabolic rates expected in hyperoxia could indeed be attributed to decreased ventilatory costs. To address the third aim (whether hyperoxia increases absolute thermal limits), the upper thermal tolerance limit (U_{TL}) of each species was assessed under hyperoxia and normoxia. U_{TL} was defined as the temperature of equilibrium loss during a standardised (2°C h^{-1}) thermal ramping exposure.

MATERIALS AND METHODS

Experimental animals and laboratory acclimation

Fish used in this study were netted from intertidal rock pools (*B. medius*) or shallow subtidal (<5 m) habitats by divers (*F. lapillum*) in the vicinity of Leigh, New Zealand. Animals were housed in 30 l flow-through seawater tanks (air saturated, 200 µm filtered, 35 ppt salinity) at the Leigh Marine Laboratory (Leigh, New Zealand) and were acclimated to a 12 h photoperiod and a temperature of 21±0.5°C for at least 4 weeks prior to experimentation. During the acclimation period, fish were fed daily on a mix of crushed aquaculture feed (Skretting, Cambridge, TAS, Australia) and pilchard. Food was withheld for a period of at least 48 h prior to the start of experimental protocols. Specific dynamic action is completed in 48 h in triplefin fish (Stobie, 2017), so a stable non-digesting state should have been reached by the time experimental protocols began. All experimental techniques were performed under approval of the University of Auckland Animal Ethics Committee (approval: 001801).

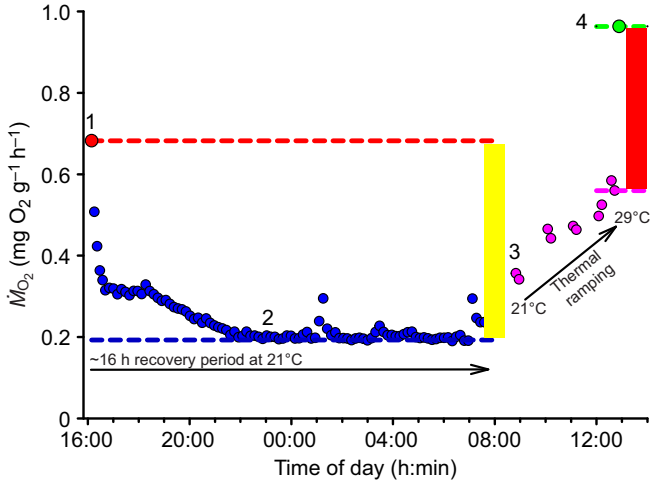

Fig. 2. Measurements of metabolic rate ($\dot{M}_{O_2}$) made in individual fish. Data points show $\dot{M}_{O_2}$ for an individual *Forsterygion lapillum* under conditions of hyperoxia (200% air saturation). The sequence of measurements shown was made in both *Bellapiscis medius* and *F. lapillum* under conditions of normoxia (100% air saturation) or hyperoxia (200% air saturation). (1) Maximum metabolic rate ($\dot{M}_{O_2,max}$) was measured at 21°C following exhaustive exercise (red circle and dashed line). (2) Standard metabolic rate (SMR) was measured at 21°C over a 16 h recovery period (blue circles and dashed line). (3) Routine $\dot{M}_{O_2}$ was measured at 21, 24, 26, 28 and 29°C under thermal ramping (pink circles). (4) $\dot{M}_{O_2,max}$ was measured at 29°C following exhaustive exercise (green circle and dashed line). The difference between $\dot{M}_{O_2,max}$ at 21°C and SMR was taken as metabolic scope at 21°C (yellow box). The difference between $\dot{M}_{O_2,max}$ at 29°C and the minimum routine $\dot{M}_{O_2}$ at 29°C was taken as the metabolic scope above routine $\dot{M}_{O_2}$ at 29°C (red box).

Experimental treatments and protocols for assessment of $\dot{M}_{O_2}$

Mass-specific oxygen consumption ($\dot{M}_{O_2}$) was assessed in *B. medius* (body mass 1.92±0.13 g, length 57±1.6 mm) and *F. lapillum* (body mass 2.13±0.11 g, length 56.4±2.7 mm) under conditions of normoxia (~21 kPa, 100% air saturation) or hyperoxia (~42 kPa, 200% air saturation) (N=10 for all treatments) and there were no differences in body mass between treatments groups (ANOVA, d.f.=3, F=1.21, P=0.32). Hyperoxia was achieved by bubbling oxygen into the seawater reservoir supplying the respirometers and was maintained at the required level by an oxy-guard control unit (Loligo Systems, Tjele, Denmark). The sequence of $\dot{M}_{O_2}$ measurements that were made in each treatment group is displayed in Fig. 2. Assessment of $\dot{M}_{O_2}$ started by transferring fish to a circular 30 l tank filled with normoxic or hyperoxic seawater at 21°C in which they were exhaustively exercised by chasing for a period of 5 min. All fish appeared to be fully exhausted after 5 min (i.e. no longer able to perform burst swimming in response to touch). At the conclusion of chasing, the fish were transferred to individual respirometry chambers (see 'Respirometry methods', below) supplied with either normoxic or hyperoxic (21°C) seawater and repeating 4 min $\dot{M}_{O_2}$ measurement cycles were initiated. $\dot{M}_{O_2,max}$ (21°C) was taken as the highest $\dot{M}_{O_2}$ value recorded in any measurement cycle, which was in all cases the first cycle following exhaustive exercise. A 5 min exhaustive exercise protocol has previously been used to elicit $\dot{M}_{O_2,max}$ values in triplefins (Khan et al., 2014a; McArley et al., 2017) and is suitable for assessment of $\dot{M}_{O_2,max}$ in benthic species which will not continuously swim in a flume (Clark et al., 2013; Norin and Clark, 2016). The fish were then left undisturbed in respirometers for a period of ~16 h under conditions of constant temperature (~21°C) and water oxygen content (normoxia or hyperoxia), and $\dot{M}_{O_2}$ was measured in repeating 10 min cycles (~100 individual $\dot{M}_{O_2}$ measurements). SMR at 21°C was defined as the mean of the lowest 10% of $\dot{M}_{O_2}$ measurements recorded in the overnight recovery period (Khan et al., 2014a; Norin et al., 2014) (Fig. 2). At ~08:00 h, the overnight SMR measurement ceased and a thermal ramping protocol was started where temperature was raised from 21°C to 29°C at a rate of ~2°C h^{-1}. Metabolic rate was measured over two measurement cycles (~15 min) at 21, 24, 26, 28 and 29°C during thermal ramping and the mean of the two measurements was defined as the temperature-specific routine $\dot{M}_{O_2}$ (Fig. 2). After the final routine $\dot{M}_{O_2}$ measurement at 29°C, the fish was removed from the respirometer and transferred to a 30 l tank filled with normoxic or hyperoxic seawater preheated to 29°C. The fish was exhaustively exercised by chasing for 5 min, then returned to the respirometer for assessment of $\dot{M}_{O_2,max}$ over four 3–4 min measurement cycles. The $\dot{M}_{O_2,max}$ at 29°C was defined as the highest value of $\dot{M}_{O_2}$ recorded over these cycles. Finally, the fish was removed from the respirometer and allowed to recover prior to recording body mass and length. The $\dot{M}_{O_2}$ measurements described above were used to determine true MS (the difference between $\dot{M}_{O_2,max}$ and SMR) under normoxia and hyperoxia at 21°C (Fig. 2). True MS could not be determined at 29°C because fish were not held at this temperature for the prolonged periods required to reliably estimate SMR. The difference between the lowest value of routine $\dot{M}_{O_2}$ at 29°C and $\dot{M}_{O_2,max}$ at 29°C was used as an approximation of MS following thermal ramping (see Fig. 2). In four individuals, routine $\dot{M}_{O_2}$ at

Journal of Experimental Biology (2018) 221, jeb189993. doi:10.1242/jeb.189993

29°C was marginally higher than $\dot{M}_{O_2,max}$ at 29°C and these individuals were treated as having zero MS above routine $\dot{M}_{O_2}$. Because MS was defined differently at 21 and 29°C, no statistical comparisons were made between temperatures.

Respirometry methods

$\dot{M}_{O_2}$, reported as milligrams of oxygen consumed per gram of body mass per hour (mg O_2 g^{-1} h^{-1}), was determined using automated intermittent-flow respirometry (Steffensen, 1989). Respirometers were constructed with a cylindrical acrylic chamber fitted with an adjustable stopper, which allowed the chamber volume (60–150 ml) to be adjusted to match fish size. The chambers were held in a 60 l reservoir filled with filtered (5 µm) UV-sterilised seawater. The temperature of the reservoir was controlled by continually pumping the seawater through a 40 l tower containing an aluminium heat exchanger. The inlet of each chamber was connected to an automated Eheim compact 3000 submersible flush pump (Eheim GmbH & Co. KG, Deizisau, Germany) that was switched on and off by a relay control unit (USB Power 8800 Pro, Aviosys International Inc., New Taipei City, Taiwan) controlled by custom-coded software (Leigh Marine Laboratory). An inline pump (modified Eheim compact 3000) was connected to the respirometry chamber in a closed loop to ensure adequate water mixing and the oxygen concentration of water within the chamber was continuously measured using contactless sensor spots and FireSting O_2 meters (PyroScience, Aachen, Germany). The decline in O_2 concentration within a respirometry chamber was used to calculate $\dot{M}_{O_2}$ in repeated measurement cycles (4–8 min) according to the equation:

$$\dot{M}_{O_2} = V\left(\frac{\Delta\%\text{sat}}{t}\right)\alpha \cdot M_b, \quad (1)$$

where V is the respirometry chamber volume minus fish volume, $\Delta\%sat/t$ is the change in oxygen saturation per unit time, α is the solubility coefficient of oxygen (mg O_2 $\%sat^{-1}$ l^{-1}) in seawater (35 ppt) and M_b is the body mass of the fish in grams (Schurmann and Steffensen, 1997). The measurement cycles were interspersed by 1 min flushing periods in order to refresh the water within the respirometers. In all estimates of metabolic rate, only $\dot{M}_{O_2}$ values with R^2 of >0.98 for the decline in oxygen per unit of time were used. The background oxygen consumption within chambers was assessed at the conclusion of thermal ramping and fish $\dot{M}_{O_2}$ was adjusted following the same method outlined in McArley et al. (2017).

Assessment of U_{TL}

U_{TL} was measured under hyperoxia (200% air saturation) and normoxia (100% air saturation) in both species (N=9–10, *B. medius*: body mass 2.03±0.24 g, length 56.6±2.1 mm, *F. lapillum*: body mass 1.52±0.12 g, length 55±1.2 mm). Assessment of U_{TL} was carried out in a 60 l reservoir filled with either normoxic or hyperoxic seawater pre-heated to 21°C. The reservoir accommodated four fish per run (~20 h) and two fish of each species were included in each run. The fish were introduced into the reservoir and allowed to acclimate overnight (~16 h) under conditions of normoxia or hyperoxia and stable temperature (~21°C). Following overnight acclimation, the temperature of the reservoir was raised at a rate of ~2°C h^{-1} until the fish lost equilibrium for a period of at least 10 s. The loss of equilibrium temperature was taken as the U_{TL} (Beitinger et al., 2000; Brijs et al., 2015; Mora and Maya, 2006; Sandblom et al., 2016). Temperature and water oxygen content were measured throughout the U_{TL} assessment using a FireSting O_2 meter (PyroScience).

Assessment of ventilation frequency, ventilation amplitude and estimated ventilation volume

Ventilation frequency and amplitude were measured by video analysis in both species under normoxia or hyperoxia and thermal ramping (21, 24, 26, 28 and 29°C) (N=8, *B. medius*: body mass 2.29±0.35 g, length 50±1.9 mm, *F. lapillum*: body mass 1.8±0.15 g, length 49.4±1 mm). There were no differences in body mass between treatment groups (ANOVA, d.f.=3, F=0.73, P=0.54). The measurements were carried out in an insulated 60×45×30 cm tank which was filled to a depth of 7 cm and continuously supplied with normoxic or hyperoxic seawater from a temperature-controlled 60 l reservoir. The depth of the tank allowed video cameras to be mounted directly above pens made of cylindrical acrylic tubing, which maintained the position of the fish throughout the measurement period. The experimental setup accommodated four fish and two of each species were used in each run. The fish were introduced into the pens and then allowed to recover overnight (~16 h) under constant temperature (~21°C) and oxygen conditions (normoxia or hyperoxia). Following overnight recovery, a thermal ramping protocol was initiated where temperature was increased from 21 to 29°C at a rate of ~2°C h^{-1} and videos were recorded at 21, 24, 26, 28 and 29°C. A single video recording (~2 min) was taken at each temperature set point and thermal ramping continued immediately after the video had been taken.

Ventilation frequency and amplitude were quantified from videos using Image J imaging software (US National Institutes of Health, Bethesda, MD, USA). Ventilation frequency was counted over 60 s in each video by using the Plot Z-axis function to measure the change in pixel intensity of an area adjacent to the operculum as it opened and closed. A 60 s time period has been used to estimate ventilation frequency in other fish species (Frisk et al., 2012). Ventilation amplitude was assessed by comparing the maximum width (mm) of the operculum when open to the width of the operculum when closed. The opening operculum was observed frame by frame during an opercula beat and width was measured in the frame where the maximum separation between the right and left operculum was observed and where the operculum was closed. Three randomly selected individual opercula beats were measured in each video and were converted to a percentage increase in operculum width. The mean of these three measurements was taken as the ventilation amplitude at each temperature. A plastic mesh grid of known dimensions attached to the base of the fish pens was used to calibrate the distance measurements in each video.

To estimate ventilation volume, the change in the volume of the head when the operculum fully opened and closed during an opercula beat was calculated. The shape of the head was treated as a half-conical frustum and volume was calculated from video frames according to the equation:

$$\text{Volume} = \frac{1}{3}\pi h(R_2^2 + R_2R_1 + R_1^2)/2, \quad (2)$$

where R_1 was equal to half the distance between the ventral tip of the right and left operculum, R_2 was equal to half the distance between each side of the fish where the operculum pivoted during a ventilatory beat and h was the distance between R_1 and R_2 along the centre of the head of the fish (Fig. 3). The difference in the calculated head volume when the operculum were opened and closed was taken as the estimated stroke volume for an opercula beat and three opercula beats were measured in each video. The mean stroke volume of three opercula beats was multiplied by ventilation frequency to estimate ventilation volume as millilitres per minute per gram of body mass. A

Journal of Experimental Biology (2018) 221, jeb189993. doi:10.1242/jeb.189993

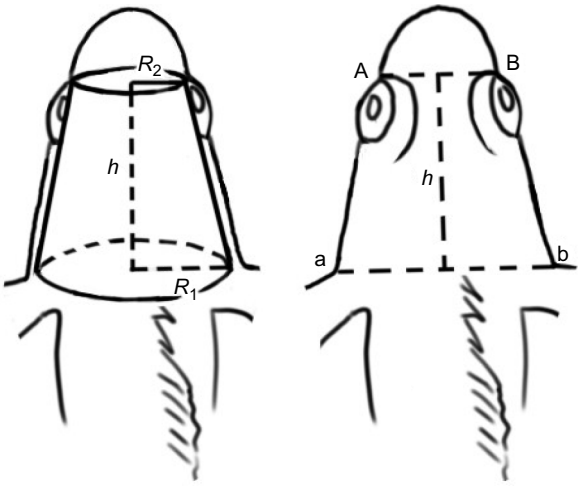

Fig. 3. Parameters measured to estimate ventilation stroke volume. The head of the fish was treated as a half-conical frustum to estimate change in volume when the opercula were opened and closed in a ventilatory beat. R_1 and R_2 were measured from video frames as half the distance between the left and right side of the fish (R_1 a–b, R_2 A–B). These points corresponded to the ventral tip of the opercula (a,b) and the point at which the opercula first pivoted in an ventilatory beat (A,B). *h*, distance between R_1 and R_2 along the centre of the head of the fish.

half-conical frustum does not completely replicate the head shape of each species. As such, estimated ventilation volume is treated as a relative measure and only compared across experimental treatments within each species.

Statistics

All statistical analysis was carried out using the SPSS Statistics 24 or Sigma Plot 13.0 software packages. Two-way analysis of variance (ANOVA) with Holm–Sidak *post hoc* comparisons was used to determine the effect of species and water oxygen content on SMR at 21°C, MS at 21°C, MS above routine $\dot{M}_{O_2}$ at 29°C and U_{TL}. For the analysis of MS above routine $\dot{M}_{O_2}$ at 29°C, the data were log transformed. The impact of species, water oxygen content (hyperoxia versus normoxia) and thermal ramping temperature on routine $\dot{M}_{O_2}$ was assessed using a three-way mixed ANOVA. For this analysis, species and water oxygen content were set as between-subjects factors and thermal ramping temperature as a within-subjects factor. For the tests of within-subjects effects, a violation of sphericity was assumed and epsilon (Huynh–Feldt)-adjusted *F*-tests were run. For all *post hoc* tests, Holm–Sidak-adjusted significance levels were used. Mixed three-way ANOVA was also used to assess the impact of species, water oxygen content and temperature on $\dot{M}_{O_2,max}$. No three-way interactions were significant ($P>0.05$) and only the two-way interactions and main effects are reported in the Results. Ventilation frequency, amplitude and estimated ventilation volume were compared within each species using repeated-measures ANOVA with temperature set as a within-subjects factor and water oxygen content set as a between-subjects factor. For *F. lapillum*, the natural logarithm of ventilation frequency was used in the analysis.

RESULTS

The impact of hyperoxia on $\dot{M}_{O_2}$

SMR was significantly higher in *B. medius* than in *F. lapillum* (ANOVA, d.f.=1, $F=44.09$, $P<0.001$) and in both species it was significantly higher under hyperoxia than under normoxia (ANOVA, d.f.=1, $F=9.03$, $P=0.005$; Fig. 4). The influence of

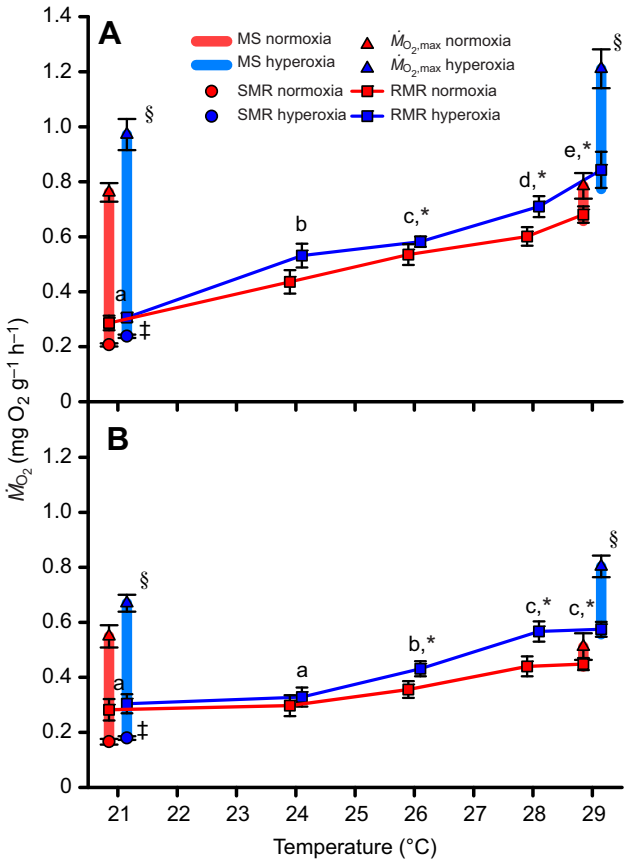

Fig. 4. $\dot{M}_{O_2}$ under normoxia and hyperoxia in triplefins. (A) *Bellapiscis medius* and (B) *Forsterygion lapillum*. All values are means±s.e.m. ($N=10$). Red and blue symbols and lines show normoxia (100% air saturation) and hyperoxia (200% air saturation), respectively. Circles show standard metabolic rate (SMR) at 21°C, triangles show maximum metabolic rate ($\dot{M}_{O_2,max}$) at 21 and 29°C, and squares show routine metabolic rate (RMR) at 21, 24, 26, 28 and 29°C under thermal ramping (note, symbols have been staggered for ease of interpretation). The thick vertical red and blue lines show metabolic scope at 21°C and scope above routine $\dot{M}_{O_2}$ at 29°C (MS). ‡Significant difference ($P<0.05$) in SMR between normoxia and hyperoxia at 21°C. §Significant difference ($P<0.05$) in $\dot{M}_{O_2,max}$ and aerobic metabolic scope between normoxia and hyperoxia at 21 and 29°C. *Significant difference ($P<0.05$) in routine $\dot{M}_{O_2}$ under thermal ramping between normoxia and hyperoxia. Lowercase letters show a significant difference ($P<0.05$) in routine $\dot{M}_{O_2}$ between thermal ramping temperatures within each species.

hyperoxia on $\dot{M}_{O_2}$ during thermal ramping was explained by an interaction between thermal ramping temperature and water oxygen content (ANOVA, d.f.=3.65, $F=3.78$, $P=0.008$). In both species, $\dot{M}_{O_2}$ was significantly higher under hyperoxia than under normoxia but only at temperatures of 26, 28 and 29°C (Fig. 4). There was also an interaction between thermal ramping temperature and species (ANOVA, d.f.=3.65, $F=12.65$, $P<0.001$). While $\dot{M}_{O_2}$ did not differ between the species at 21°C, at temperatures of 24, 26, 28 and 29°C, *B. medius* had a significantly higher $\dot{M}_{O_2}$ than *F. lapillum*. Moreover, $\dot{M}_{O_2}$ increased significantly from 21 to 24°C and from 28 to 29°C in *B. medius* (Fig. 4A), but there was no significant difference in $\dot{M}_{O_2}$ over these temperature changes for *F. lapillum* (Fig. 4B).

The impact of species, temperature and water oxygen content on $\dot{M}_{O_2,max}$ was explained by interactions between temperature and species (ANOVA, d.f.=1, $F=4.15$, $P=0.049$), and temperature and water oxygen content (ANOVA, d.f.=1, $F=22.4$, $P<0.001$).

Journal of Experimental Biology (2018) 221, jeb189993. doi:10.1242/jeb.189993

Under all conditions, $\dot{M}_{O_2,max}$ was significantly higher in *B. medius* than in *F. lapillum* but the difference between the species was greatest at 29°C (Fig. 4). $\dot{M}_{O_2,max}$ was significantly higher under hyperoxia than under normoxia in all treatments; however, the difference in $\dot{M}_{O_2,max}$ between hyperoxia and normoxia was larger at 29°C than at 21°C (Fig. 4). Interestingly, in both species, $\dot{M}_{O_2,max}$ increased significantly between 21 and 29°C under hyperoxia whereas under normoxia there was no change in $\dot{M}_{O_2,max}$ between these temperatures (Fig. 4).

At 21°C, MS was significantly greater under hyperoxia in both species (ANOVA, d.f.=1, F=13.12, P<0.001) and higher in *B. medius* than in *F. lapillum* (ANOVA, d.f.=1, F=27.71, P<0.001) (Fig. 4). Following thermal ramping to 29°C, MS above routine $\dot{M}_{O_2}$ was significantly higher under hyperoxia than under normoxia in both species (Fig. 4). *Bellapiscis medius* had significantly greater MS above routine $\dot{M}_{O_2}$ than *F. lapillum* at 29°C (ANOVA, d.f.=1, F=5.46, P=0.025).

U_{TL}

There was a significant interaction between the effect of species and water oxygen content on U_{TL} (ANOVA, d.f.=1, F=4.19, P=0.048). Although the differences were small (<0.6°C), the intertidal specialist *B. medius* had a significantly higher U_{TL} than *F. lapillum* under both normoxia and hyperoxia (Fig. 5). There was no effect of hyperoxia on U_{TL} in *B. medius* but U_{TL} did increase (0.43°C) under hyperoxic conditions in *F. lapillum* (Fig. 5).

Ventilation frequency, ventilation amplitude and estimated ventilation volume

In *B. medius* and *F. lapillum*, ventilation frequency during thermal ramping was significantly lower under hyperoxia than under normoxia (*B. medius*: ANOVA, d.f.=1, F=13.74, P=0.002 and *F. lapillum*: ANOVA, d.f.=1, F=10.65, P=0.006; Fig. 6A) but there was no effect of hyperoxia on ventilation amplitude (Fig. 6B). Estimated ventilation volume during thermal ramping was also significantly lower under hyperoxia in both species (*B. medius*: ANOVA, d.f.=1, F=44.1, P<0.001 and *F. lapillum*: ANOVA, d.f.=1, F=47.93, P<0.001; Fig. 6C). During thermal ramping, ventilation frequency increased significantly at higher temperatures in *B. medius* (ANOVA, d.f.=4, F=11.44, P<0.001; Fig. 6Ai) but there was no effect of temperature in *F. lapillum* (ANOVA, d.f.=4, F=1.04, P=0.37; Fig. 6Aii). Contrastingly, there was no change in ventilation amplitude across temperature in *B. medius* (ANOVA, d.f.=4, F=0.96, P=0.43; Fig. 6Bi) but, in *F. lapillum*, ventilation amplitude increased at higher temperatures (ANOVA, d.f.=4, F=28, P<0.001; Fig. 6Bii). In both species, estimated ventilation volume increased with thermal ramping (*B. medius*: ANOVA, d.f.=4, F=21.98, P<0.001 and *F. lapillum*: ANOVA, d.f.=4, F=29.9, P<0.001; Fig. 6C).

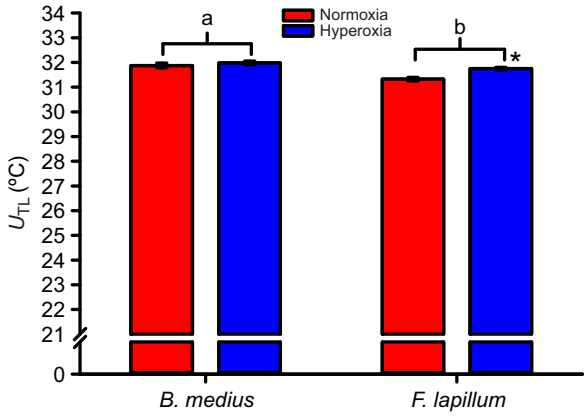

Fig. 5. Upper thermal tolerance limit (U_{TL}) in normoxia- and hyperoxia-exposed *B. medius* and *F. lapillum*. All values are means±s.e.m. (N=9–10). Red and blue bars show normoxia (100% air saturation) and hyperoxia (200% air saturation), respectively. *Significant difference (P<0.05) between water oxygen content within species. Lowercase letters show a significant difference between species.

DISCUSSION

This study shows that hyperoxia increases $\dot{M}_{O_2,max}$ and MS in intertidal triplefin fish. $\dot{M}_{O_2,max}$ was higher with hyperoxia than under normoxia at ambient temperature (21°C) and also after thermal ramping to ecologically relevant temperatures (29°C). Consequently, despite higher SMR and routine $\dot{M}_{O_2}$, MS at 21°C and MS above routine $\dot{M}_{O_2}$ at 29°C increased under hyperoxia. At the start of thermal ramping events in rock pools, intertidal fish are first exposed to normoxia and ambient temperature, but, as temperature increases, rock pools can also become super-saturated with oxygen (see Fig. 1B for an example). Therefore, comparisons of MS between ambient temperature normoxic conditions and high-temperature hyperoxic conditions are relevant. In hyperoxia, 79% and 64% of the MS available under normoxia and ambient temperature (21°C) was retained after thermal ramping to 29°C in *B. medius* and *F. lapillum*, respectively. This contrasts with the near-complete collapse of MS in both species at 29°C under normoxia or, more specifically, without hyperoxia (Fig. 4). While hyperoxia did not provide a meaningful increase in the absolute thermal tolerance limits of either species, the observed retention of MS may allow intertidal fish to continue performing aerobically demanding activities, such as feeding and digestion, to higher temperatures than would otherwise be possible. These findings indicate that environmental hyperoxia may provide a 'metabolic refuge' for intertidal fish exposed to acute thermal stress during low tide ebbs if photosynthesis is active.

The influence of hyperoxia on aerobic metabolic scope

MS is thought to be a key determinant of fitness as it represents the boundaries within which aerobic activities can be performed (Claireaux and Lefrancois, 2007; Clark et al., 2013). Indeed, positive correlations between growth performance and MS have been demonstrated in several fish species (Claireaux et al., 2000; Claireaux and Lefrancois, 2007; Jobling, 1981; Khan et al., 2014b), including the triplefin *F. lapillum* (McArley et al., 2017). If increased availability of MS does indeed help to mitigate a prioritisation or constraint on simultaneous energetic processes (Claireaux and Lefrancois, 2007; Clark et al., 2013; Lefrançois and Claireaux, 2003), then an expansion of MS under hyperoxia may offset expected losses in performance during acute high-temperature exposure. For example, if rock pools become hyperoxic and MS is expanded, feeding and digestion may be better supported at high temperatures, despite increased basal costs. Interestingly, Hilton et al., (2008) showed that the intertidal triplefin *B. medius* was more abundant in rock pools with a high algal coverage, suggesting a preference for rock pools likely to become hyperoxic during low tide. Greater MS under hyperoxia may also allow rock pool fish to avoid an anaerobic acidosis, which may develop during thermal ramping as anaerobic metabolism increases to meet ATP demands (e.g. Clark et al., 2008). Indeed, Devor et al. (2016) found that hyperoxia mitigated the rise in plasma and muscle lactate levels in two Antarctic notothenioid fishes exposed to

Journal of Experimental Biology (2018) 221, jeb189993. doi:10.1242/jeb.189993

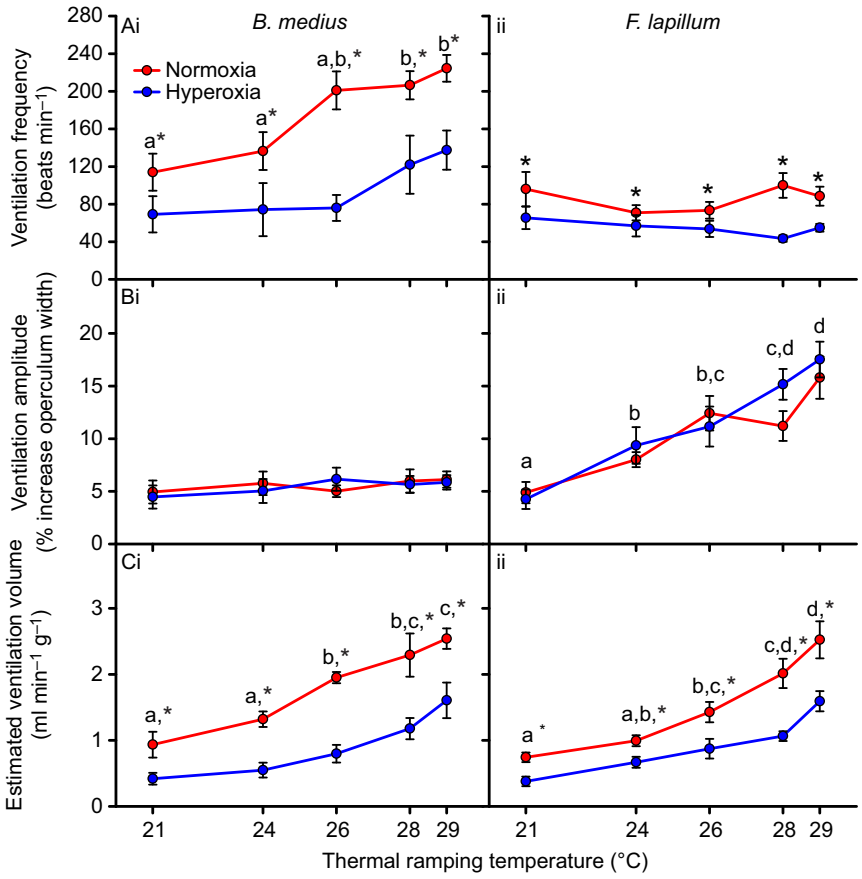

Fig. 6. Ventilation frequency, amplitude and volume during thermal ramping in normoxia- and hyperoxia-exposed *B. medius* and *F. lapillum*. (A) Ventilation frequency, (B) ventilation amplitude and (C) estimated ventilation volume for (i) *B. medius* and (ii) *F. lapillum*. All values are means±s.e.m. (N=8). Red and blue symbols and lines show normoxia (100% air saturation) and hyperoxia (200% air saturation), respectively. *Significant difference (P<0.05) between normoxia and hyperoxia during thermal ramping. Lowercase letters show a significant difference (P<0.05) between thermal ramping temperatures.

thermal ramping. So, whether hyperoxia helps intertidal fish recover from heat stress by avoiding a metabolic acidosis or whether hyperoxia assists other aspects of performance (feeding, digestion, growth, etc.) during thermal ramping would of course be interesting avenues for future research.

The exclusively intertidal triplefin *B. medius* has a higher MS than *F. lapillum*, a species found more commonly in shallow subtidal habitats. *Bellapiscis medius* is also found in higher intertidal zone rock pools than *F. lapillum* (T.J.M., personal observation) and occupies habitat more prone to acute high-temperature events and, possibly, night-time hypoxia too. Whether it is increased basal metabolic costs during thermal ramping or constrained maximum metabolic capacity during hypoxia, intertidal fish regularly face environmental conditions that limit MS. The high MS of *B. medius* may therefore reduce the occurrence of situations where limiting environmental conditions result in the prioritisation or constraint of aerobic activities (e.g. growth, reproduction, swimming, etc.).

Possible mechanisms by which aerobic metabolic scope increases under hyperoxia

In the current study, SMR and routine $\dot{M}_{O_2}$ both increased under hyperoxic conditions but not to the extent that $\dot{M}_{O_2,max}$ increased. Thus, the observed increase in MS under hyperoxia was driven primarily by the response of $\dot{M}_{O_2,max}$ and this is in line with the observations of Brijs et al. (2015), who found that the MS of European perch approximately double at 23°C under hyperoxia (~200% air saturation) as a result of expansion in $\dot{M}_{O_2,max}$. This is not universal among all fish species, however, because hyperoxia did not increase $\dot{M}_{O_2,max}$ or MS in either rainbow trout (Duthie and Hughes, 1987) or common sole (Lefrançois and Claireaux, 2003). Brijs et al., (2015) also suggested a higher $\dot{M}_{O_2,max}$ under hyperoxia at temperatures approaching thermal limits (~34°C) but the results of the present study are the first to directly demonstrate an increase in $\dot{M}_{O_2,max}$ under hyperoxia following thermal ramping.

So, if MS expansion under hyperoxia results from increased $\dot{M}_{O_2,max}$, how does this expansion occur? The oxygen consumption rate of an intact animal is the sum of aerobic metabolic demands of all tissues, which is mostly determined by the oxygen carrying capacity of blood and the rate at which the circulatory system delivers oxygen to respiring tissues. While hyperoxia probably increases $\dot{M}_{O_2,max}$ by modulating these factors, the exact mechanisms by which this occurs remain unclear. European perch lacks a coronary circulation and, therefore, relies on the venous blood return to supply oxygen to the heart (Ekström et al., 2016; Santer and Walker, 1980). Although we do not know whether triplefin fish also lack a coronary circulation, it is not present in another blennioid fish species (*Blennius pholis*) (Santer and Walker, 1980). If triplefin fish do indeed lack a coronary circulation like European perch, one possibility is that cardiac output is improved under hyperoxia following exhaustive exercise as a result of an increased supply of oxygen to the heart via the venous blood return. Indeed, elevated partial pressure of oxygen in venous blood has been demonstrated in fish exposed to hyperoxia (Ekström et al., 2016; Takeda, 1990; Wilkes et al., 1981). This could also explain why there was no effect of hyperoxia on $\dot{M}_{O_2,max}$ in rainbow trout (*Oncorhynchus mykiss*) (Duthie and Hughes, 1987) because salmonids possess a well-developed coronary circulation which supplies the heart directly with oxygenated blood (Ekström et al., 2017; Santer and Walker, 1980) and this could theoretically override the influence of hyperoxia on venous blood oxygen content and hence cardiac output. An alternative suggestion is that cutaneous oxygen uptake is increased in hyperoxia (Brijs et al., 2015; Ekström

et al., 2016; Wang et al., 2014). There is evidence that the contribution of the skin to gas exchange is upregulated following exercise in fish (Rummer et al., 2014) and cutaneous respiration provides a relatively greater contribution to the total $\dot{M}_{O_2}$ of carp (*Cyprinus carpio*) under hyperoxia (Takeda, 1989).

While increased $\dot{M}_{O_2,max}$ in hyperoxia may be beneficial for rock pool fish through enabling MS expansion, hyperoxia does appear to elevate basal metabolic costs. Here, we hypothesised that basal metabolic costs decrease under hyperoxia, yet the SMR at ambient temperature (21°C) and routine $\dot{M}_{O_2}$ at high temperatures (26–29°C) during thermal ramping were higher under hyperoxia than under normoxia. This observation is somewhat paradoxical because, as expected, our measures showed that ventilation effort (and presumably the energetic cost of ventilation) was decreased with hyperoxia (Fig. 6). This cost pattern was also apparent for the rainbow trout, which showed a transient increase in $\dot{M}_{O_2}$ despite a 50% reduction in ventilatory flow rate under hyperoxia (Wood and Jackson, 1980), and spotted wolfish (*Anarhichas minor*) also showed higher routine $\dot{M}_{O_2}$ under hyperoxia (Foss et al., 2003). A similar decrease in ventilation with hyperoxia has also been seen in carp (Takeda, 1990), the white sucker (*Catostomus commersonii*) (Wilkes et al., 1981) and an intertidal goby (*Gobius cobitis*) (Berschick et al., 1987), but in these species $\dot{M}_{O_2}$ was unchanged, suggesting a relatively smaller increase in basal costs with hyperoxia. Taken together, these findings suggest the basal metabolic costs of fish are raised under hyperoxia and these costs appear to override the reduced costs of ventilation. While higher basal metabolic costs under hyperoxia put a performance benefit of hyperoxia in question, in the current study they occurred alongside comparatively larger increases in $\dot{M}_{O_2,max}$, such that MS was still expanded with hyperoxia. Clearly, further work is required to determine whether intertidal fish facing thermal ramping are able to derive a performance benefit from expanded MS under hyperoxia.

The influence of hyperoxia on absolute U_{TL}

Despite MS increasing at temperatures approaching critical limits, hyperoxia did not provide a biologically relevant increase in the absolute thermal limits of either triplefin species. This finding is in agreement with other recent studies, which found no or only a small increase in the critical thermal maximum of fish under hyperoxia (Brijs et al., 2015; Devor et al., 2016; Ekström et al., 2016; Healy and Schulte, 2012a). Furthermore, the U_{TL} of *B. medius* was only 0.55 and 0.23°C higher than that of *F. lapillum* under normoxia and hyperoxia, respectively, even though $\dot{M}_{O_2,max}$ in *B. medius* was substantially higher (32–42% greater $\dot{M}_{O_2,max}$) under all conditions. These findings therefore contribute to a growing body of literature suggesting that the absolute thermal limits of fish are not limited by the ability of the cardio-respiratory system to deliver oxygen to tissues (Brijs et al., 2015; Devor et al., 2016; Ekström et al., 2016; Ern et al., 2016; Healy and Schulte, 2012a; Wang et al., 2014). These findings also suggest that the constraints that set the absolute thermal limits of these species develop at a similar temperature under normoxia and hyperoxia. What these constraints are and when they develop in relation to loss of equilibrium is unknown, but beyond this temperature, any expansion of MS under hyperoxia would probably be inconsequential as death would rapidly ensue.

The most extreme rock pool temperature observed over a 3 week period in summer was 30.1°C (Fig. 1A). While this temperature is below the U_{TL} of each triplefin fish species, it is within 2°C of their absolute thermal limits even under hyperoxic conditions. Moreover, the rate of temperature increase used to determine U_{TL} (2°C h^{-1}) was faster than the rate at which peak temperatures observed in rock pools were reached (1.3°C h^{-1}). Therefore, as U_{TL} is often reduced under slower rates of warming (Mora and Maya, 2006), the absolute thermal limits of these species may be even closer to observed maximum rock pool temperatures under fully replicated rock pool conditions. This presents a cause for concern because climate change is expected to result in more frequent and extreme transient heat waves (Perkins et al., 2012), which could push temperature maximums of many rock pools beyond the tolerance limits of temperate intertidal triplefin fishes. In this scenario, intertidal fishes may experience habitat loss and increased risk of overheating, unless intergenerational adaption can select for more thermally tolerant phenotypes.

A limitation of the current study was that both species were acclimated to constant environmental conditions prior to the assessment of U_{TL}. In the wild, rock pool fish are exposed to constantly fluctuating physico-chemical conditions which could potentially condition U_{TL} beyond the levels observed in the present investigation. The influence of prior exposure to fluctuating temperature and other relevant environmental conditions on U_{TL} in intertidal fishes should be considered in future studies.

Conclusions

In the intertidal triplefins *B. medius* and *F. lapillum*, hyperoxia increased $\dot{M}_{O_2,max}$ and MS both at ambient temperature and following acute thermal ramping to temperatures observed in rock pools. This finding suggests that algae-mediated hyperoxia in rock pools mitigates the collapse of MS in intertidal fish which would otherwise occur under normal air saturated oxygen conditions and thermal ramping. Retention of MS under hyperoxia offers several potential benefits to intertidal fish facing acute high-temperature exposure including less severe constraint of aerobically demanding activities and mitigation of anaerobic stress. However, as hyperoxia did not meaningfully increase the absolute thermal limits of either species, further studies are required to establish whether there are benefits of hyperoxia for intertidal fish, and at what temperatures they occur relative to loss of equilibrium. The U_{TL} of both *B. medius* and *F. lapillum* was less than 2°C above maximal observed rock pool temperatures, even with hyperoxia. This confirms that these species, particularly the exclusively intertidal *B. medius*, reside in a habitat where they are periodically exposed to temperatures close to their thermal tolerance limits. With more extreme heat waves predicted with climate change, intertidal triplefins may face loss of habitat and increased risk of heat death as rock pool temperature maximums increase. With this understanding, a comprehensive analysis of the likely impact of climate change on the availability of thermally suitable rock pool habitats is warranted.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.J.M., N.A.H.; Methodology: T.J.M., N.A.H.; Formal analysis: T.J.M.; Investigation: T.J.M.; Writing - original draft: T.J.M.; Writing - review & editing: T.J.M., A.J.R.H., N.A.H.; Supervision: A.J.R.H.; Funding acquisition: A.J.R.H.

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