Journal of Comparative Physiology B (2019) 189:399–411
https://doi.org/10.1007/s00360-019-01216-w

ORIGINAL PAPER

Intertidal triplefin fishes have a lower critical oxygen tension (P_{crit}), higher maximal aerobic capacity, and higher tissue glycogen stores than their subtidal counterparts

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Received: 22 October 2018 / Revised: 16 March 2019 / Accepted: 20 March 2019 / Published online: 2 April 2019

Abstract

Decreased oxygen (O_2) availability (hypoxia) is common in rock pools and challenges the aerobic metabolism of fishes living in these habitats. In this study, the critical O_2 tension (P_{crit}), a whole animal measure of the aerobic contribution to hypoxia tolerance, was compared between four New Zealand triplefin fishes including an intertidal specialist (*Bellapiscis medius*), an occasional intertidal inhabitant (*Forsterygion lapillum*) and two exclusively subtidal species (*F. varium* and *F. malcolmi*). The intertidal species had lower P_{crit} values than the subtidal species indicating traits to meet resting O_2 demands at lower O_2 tensions. While resting O_2 demand (standard metabolic rate; SMR) did not show a major difference between species, the intertidal species had higher maximal rates of O_2 consumption ($\dot{M}O_{2,max}$) and higher aerobic metabolic scope (MS). The high O_2 extractive capacity of the intertidal species was associated with increased blood O_2 carrying capacity (i.e., higher Hb concentration), in addition to higher mass-specific gill surface area and thinner gill secondary lamellae that collectively conveyed a higher capacity for O_2 flux across the gills. The specialist intertidal species *B. medius* also had higher glycogen stores in both white muscle and brain tissues, suggesting a greater potential to generate ATP anaerobically and survive in rock pools with O_2 tensions less than P_{crit}. Overall, this study shows that the superior P_{crit} of intertidal triplefin species is not linked to a minimisation of SMR, but is instead associated with an increased O_2 extractive capacity of the cardiorespiratory system (i.e., $\dot{M}O_{2,max}$, MS, Hb and gill O_2 flux).

Keywords Hypoxia · P_{crit} · Intertidal fish · Aerobic scope · Aerobic metabolism · Gills

Abbreviations

ACD	Above chart datum
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
ATP	Adenosine triphosphate
CO_2	Carbon dioxide
Hb	Haemoglobin
Hct	Haematocrit
$\dot{M}O_2$	Mass-specific oxygen consumption
$\dot{M}O_{2,max}$	Maximum aerobic metabolic rate post-exhaustive exercise
MS	Aerobic metabolic scope
O_2	Oxygen
P_{crit}	Critical oxygen tension
PO_2	Oxygen tension
RBC	Red blood cell
SGA	Sectioned gill arch
SL	Gill secondary lamellae
SMR	Standard metabolic rate
WGA	Whole intact gill arch

Communicated by H. V. Carey.

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Introduction

To carry out the necessary requirements of life, all aerobic organisms need to acquire enough O_2 to drive ATP production via oxidative phosphorylation. Therefore, decreased O_2 availability (environmental hypoxia), which is a common

occurrence in a range of aquatic habitats, presents as a challenge for organisms such as fish (Diaz and Breitburg 2009; Farrell and Richards 2009). Intertidal fish can be exposed to hypoxic conditions when rock pool organisms consume O_2 at a faster rate than it can be replaced by either algal photosynthesis or diffusion from air (Berschick et al. 1987; Bridges 1988; Richards 2011; Truchot and Duhamel-Jouve 1980). Thus, hypoxia is prevalent in rock pools at night when there is no photosynthetic activity to buffer O_2 depletion due to respiration (Table 1; Fig. 1A, B). Well-mixed coastal subtidal habitats on the other hand have relatively stable O_2 conditions (Fig. 1C) and, while hypoxia can develop in subtidal habitats (e.g., due to eutrophication and algal blooms or poor mixing), the frequent and large change in O_2 availability typical of rock pools does not occur to the same extent. Notably, comparisons of closely related intertidal and subtidal species have proven to be a useful system in which to examine physiological mechanisms of hypoxia tolerance in fish (Brix et al. 1999; Hilton et al. 2010; Mandic et al. 2012; Mandic et al. 2009; Richards 2011; Speers-Roesch et al. 2013).

Environmental hypoxia represents a continuum of diminishing O_2 availability under which it becomes increasingly challenging for organisms to take up adequate O_2 to meet energetic demands via aerobic metabolism. If O_2 availability is critically low, organisms cannot extract enough O_2 to meet even basal energetic demands aerobically and O_2 independent (anaerobic) ATP production is recruited to maintain energy balance (Richards 2011). O_2 independent ATP production, however, is relatively inefficient, dependent on a finite pool of endogenous fermentable fuels (e.g., glycogen) and results in the accumulation of by-products (e.g., ADP, lactate and H^+) which can be deleterious (Nilsson and Östlund-Nilsson 2008; Richards et al. 2007; Richards 2011; Speers-Roesch et al. 2013). Thus, a key strategy for hypoxia survival is to continue to meet O_2 demand even at low environmental O_2 tensions (PO_2) (Mandic et al. 2009). In fish, the capacity to meet O_2 demand under hypoxia can be measured as the critical PO_2 (P_{crit}), which is defined as

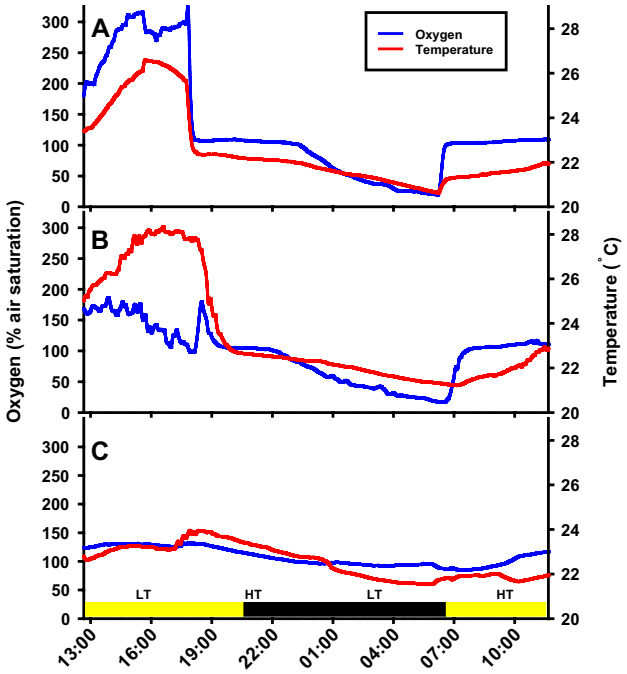

Fig. 1 Changes in oxygen availability (% of air saturation) and temperature (°C) in two rock pools inhabited by intertidal triplefin fish (**A**, **B**) and at a shallow subtidal site (**C**). Values represent measurements logged every 5 min between the 5th and 6th of February 2019. *LT* low tide, *HT* high tide. Yellow and black shaded areas in panel C show daytime and nighttime hours, respectively. The two intertidal rock pools were located at Goat Island, Leigh, New Zealand (36°16′S, 174°47′E) and the subtidal site was located immediately adjacent to the rock pools at a depth of ~2 m (color figure online)

Table 1 Oxygen (O_2) concentration, temperature and fish species observed in ten rock pools approximately 1 h after low tide at Hatfield's Beach, Auckland, New Zealand (36°34′S, 174°41′E)

Pool	O_2 content (% air saturation)		Temperature (°C)		Species observed	
	Day	Night	Day	Night	Day	Night
1	109.1	26.3	25.5	22.2	Bm	Bm
2	41.6	10.3	23.9	21.5	Bm, Af	Af
3	95.2	31.7	23.9	21.7	Bm, Af	Bm, Af
4	87.2	16	23.2	21.3	na	Bm
5	87.1	35.6	22.6	20.6	Bm, Fl	Bm, Fl
6	113.2	55.3	22.6	20.3	na	Bm
7	113.1	48.8	22.6	21.9	na	Bm, Fl, Af
8	43.9	13.1	22.7	20.8	na	Bm, Af
9	51.3	9.4	24.6	21.4	na	na
10	196.8	17.3	26.9	21.7	Bm	Bm

Values represent spot measurements taken at a mid-level depth in the rock pools between 0530 and 0630 h (nighttime low tide 0423 h) and 1730–830 h (daytime low tide 1649 h) on the 26th of February 2016

Bm, *Bellapiscis medius*; Af, *Acanthoclinus fuscus* (Jenyns, 1842), Fl, *Forsterygion lapillum*

the lowest O_2 tension at which a resting O_2 consumption rate ($\dot{M}O_2$) can be maintained. In practical terms, P_{crit} is the PO_2 at which $\dot{M}O_2$ is forced lower than standard metabolic rate (SMR) (Claireaux and Chabot 2016; Rogers et al. 2016). Importantly, fish exposed to PO_2 equivalent to 30% of their known P_{crit} accumulate lactate, deplete tissue glycogen and ATP stores and eventually lose equilibrium (Speers-Roesch et al. 2013). In the sculpins, intertidal species with a lower P_{crit} survive extreme hypoxia for longer (Mandic et al. 2012), showing that a low P_{crit} makes an important contribution to overall hypoxia tolerance in at least some intertidal fishes. Theoretically, species with a lower P_{crit} should be more hypoxia tolerant as a high extractive capacity for O_2 allows energy demand to be met through the efficient aerobic pathway at lower PO_2, thus avoiding or limiting the extent to which a time-limited anaerobic strategy must be relied upon for survival. It should be acknowledged, however, that some fishes can be very hypoxia tolerant and also have a relatively high P_{crit}. This phenomenon is seen in the Amazonian oscar (*Astronotus ocellatus*) which can survive up to 6 h anoxia at 28 °C but has a P_{crit} of 6.1 kPa (Scott et al. 2008). However, rather than relying on a high extractive capacity for O_2, the Amazonian oscar is thought to achieve hypoxia tolerance through a combination of metabolic rate depression and a high tolerance of the end-products of anaerobic metabolism (Scott et al. 2008).

According to Mandic et al. (2009), intertidal sculpins have a lower P_{crit} than their subtidal counterparts, indicating that the ability to meet resting O_2 demand at low PO_2 has been selected for fishes occupying intertidal habitats with variable O_2 conditions. Mandic et al. (2009) also showed that sculpin species with a low P_{crit} have red blood cells (RBC) with a higher affinity to bind O_2 (low whole RBC P_{50}), a larger mass-specific gill surface area and a lower routine $\dot{M}O_2$. Thus, it appears that a low P_{crit} requires a high extractive capacity for O_2, which can then also be put to use to meet a low basal requirement for O_2. Therefore, one prediction is that species with a lower P_{crit} will have a cardiorespiratory system with a high capacity to take up O_2 (i.e., a high maximum metabolic rate [$\dot{M}O_{2,max}$]) and also a low SMR (the best estimate of resting O_2 demand in a recovered post-absorptive animal). This combination of respiratory characteristics would mean that species with a lower P_{crit} will also have higher aerobic metabolic scope (MS), representing the difference between $\dot{M}O_{2,max}$ and SMR.MS reflects the capacity of an organism to perform O_2 demanding activities (e.g., growth, activity, feeding, etc.) beyond maintenance requirements and a constraint upon MS has been proposed as a crucial determinant of performance in fish facing hypoxia (Chabot and Claireaux 2008; Claireaux and Chabot 2016; Claireaux and Lefrancois 2007). Intertidal rock pool fish with a high MS might therefore avert impaired aerobic performance during rock pool hypoxia (Fig. 1; Table 1), but MS has yet to be compared among intertidal and subtidal fishes.

New Zealand triplefins (family: Tripterygiidae) are a highly specious and closely related group of marine blennoid fishes (Hickey and Clements 2005) which show habitat partitioning by depth and exposure across the nearshore coastal environment (Wellenreuther et al. 2007). Comparisons among New Zealand triplefin fishes already show that intertidal species have a lower P_{crit} than subtidal species (Hilton et al. 2008, 2010; Hilton 2010). However, it is possible that the protocols and respirometry methods used in this previous work produced elevated estimates of P_{crit}. For example, fish within these studies were allowed to recover for only 2.5 h within respirometers prior to hypoxia exposure and P_{crit} measurement. The problems associated with the use of a short recovery time when estimating P_{crit} are twofold. First, a reliable estimate of resting $\dot{M}O_2$ (i.e., SMR) may be precluded and, if this is the case, P_{crit} has to be identified as the breakpoint in $\dot{M}O_2$ during hypoxia exposure using segmented regression. Segmented regression is not recommended for P_{crit} determination because, if for any reason the $\dot{M}O_2$ of fish is elevated at the time of hypoxia exposure (e.g., due to incomplete recovery from handling stress or spontaneous activity), this methodology will overestimate P_{crit} (Claireaux and Chabot 2016). This problem is exacerbated when making comparisons of hypoxia tolerance among multiple species that may recover from handling stress at different rates, and/or be more or less active within respirometers. Furthermore, previous estimates of P_{crit} in triplefins (i.e., Hilton et al. 2008; Hilton 2010; Hilton et al. 2010) were made using the closed-system respirometry, which in some cases has been demonstrated to confound $\dot{M}O_2$ measurements due to the accumulation of waste products (e.g., CO_2) within respirometers (Rogers et al. 2016; Snyder et al. 2016). Therefore, in the present investigation, we determined it necessary to re-examine the P_{crit} of triplefin species, but this time using intermittent stop-flow respirometry (Steffensen 1989) and more established methods for P_{crit} measurement (see Claireaux and Chabot 2016; Snyder et al. 2016).

In this study, the capacity to meet resting O_2 demands under hypoxia (i.e., P_{crit}) was compared among four New Zealand triplefin fish species (*Bellapiscis medius*, *Forsterygion lapillum, F. varium* and *F. malcolmi*). These four species were included as they occupy different habitat depths (intertidal to subtidal; Table 2) and represented a broad range of P_{crit} for this group (i.e., low, medium and high P_{crit}) in a previous investigation (Hilton 2010). *B. medius* is an intertidal specialist which occupies rock pools located on the shoreline, on average 1.39 m (range 0.31–3.39 m) above chart datum (ACD) (Hilton et al. 2008). *F. lapillum* is an occasional occupant of intertidal rock pools, but is most abundant in relatively shallow (mean depth of occurrence 3.5 m; Table 2) subtidal habitats. In the intertidal

Table 2 Habitat depth, gill morphometry, tissue glycogen and haematological parameters in *B. medius*, *F. lapillum*, *F. varium* and *F. malcolmi* ($N=8$ for gill morphometry, $N=10$ for tissue glycogen and $N=8$–10 for haematology)

Trait	*B. medius*	*F. lapillum*	*F. varium*	*F. malcolmi*
Habitat depth range (m)	0–2	0–10	0–30	5–35
Mean depth of occurrence (m)	na	3.5	8	11
Gill morphometry				
Body mass (g)	4.33 (0.29)	2.17 (0.12)	11.28 (1.23)	9.09 (0.94)
Filament number (no. arch^{-1})	50.6 (0.89)^{a}	44.5 (0.87)^{b}	54.2 (1.52)^{a}	52.3 (0.85)^{a}
Filament length (mm g BM^{-1})	129.2 (7.7)^{a}	171.7 (7)^{a}	65.1 (5)^{b}	72.2 (3.4)^{b}
SL density (no. mm^{-1} filament)	26.3 (0.33)^{a}	25.6 (0.48)^{a}	17 (0.4)^{b}	16.7 (0.63)^{b}
SL SA (mm^2)	0.05	0.05	0.14	0.14
SL thickness (μm)	6.9 (0.26)^{a}	7.9 (0.19)^{b}	10.4 (0.21)^{c}	11.2 (0.5)^{c}
SL height (μm)	104.1 (2.5)^{a}	98.9 (2.7)^{a}	146.5 (4.8)^{b}	148.7 (8.4)^{b}
SL basal length (μm)	233.5 (5.4)^{a}	245.5 (4.9)^{a}	401.6 (11.4)^{b}	418.8 (18.4)^{b}
Mass-specific SA (mm^2 g BM^{-1})	364.9 (18.3)^{a}	475.3 (16.8)^{b}	315.9 (30.2)^{a}	328.3 (13.5)^{a}
Gill $\dot{V}O_2$ (μM O_2 g^{-1} h^{-1})	114.2 (7.5)^{a}	128.8 (6.9)^{a}	65.5 (6.4)^{b}	63.3 (2.8)^{b}
Tissue glycogen				
Brain glycogen (μM g ww^{-1})	4.47 (0.46)^{a}	3.16 (0.53)^{b}	2.54 (0.14)^{b}	2.39 (0.17)^{b}
WM glycogen (μM g ww^{-1})	52.8 (5.58)^{a}	22.3 (3.35)^{b}	16.8 (2.96)^{bc}	10.2 (2.32)^{c}
HSI	5.77 (0.34)^{a}	3.43 (0.34)^{c}	4.95 (0.45)^{ab}	3.96 (0.45)^{bc}
Liver glycogen (μM g ww^{-1})	375.8 (28)	346.1 (28.6)	360.7 (21.6)	335.6 (37.9)
Liver glycogen (μM g BM^{-1})	22.08 (2.46)^{a}	12.13 (1.79)^{b}	18.07 (2.1)^{ab}	14.4 (2.71)^{ab}
Haematology				
Hb (g L^{-1})	95.5 (2.52)^{a}	91.9 (3.17)^{a}	83.3 (3.68)^{ab}	75.6 (4.62)^{b}
Hct (%)	22.9 (0.84)	21.5 (1.38)	21.4 (1.28)	23.4 (1.92)

Values are means with SEM in parenthesis

Significant differences ($P<0.05$) between species are shown by lower case letters not shared

Species depth ranges taken from Hilton (2010) and references therein. Mean depth of occurrence taken from Wellenreuther et al. (2007). Note *B. medius* was not included in the subtidal surveys carried out by Wellenreuther et al. (2007) and this species appears to be exclusively intertidal (Hilton et al. 2008)

BM body mass, *SL* secondary lamellae, *SA* surface area, *WM* white muscle, *HSI* hepatosomatic index, *Hb* haemoglobin, *Hct* haematocrit

zone, *F. lapillum* is less abundant than *B. medius*, however, their distributions, at least in terms of shoreline elevation, do overlap as *F. lapillum* is found in rock pools on average 1.21 m (range 0.19–3.09 m) ACD (Wellenreuther 2007). *Forsterygion varium* and *F. malcolmi* are both exclusively subtidal species with mean depth of occurrences of 8 and 11 m, respectively (Table 2). The inclusion of these four species allowed the physiological characteristics of a specialist intertidal species (*B. medius*) to be compared to three other relatively closely related triplefin species which are either occasionally intertidal (*F. lapillum*) or exclusively subtidal (*F. varium* and *F. malcolmi*). Additionally, we could compare the physiological characteristics of the occasional intertidal rock pool inhabitant *F. lapillum* to that of two exclusively subtidal species from the same genus (*F. varium* and *F. malcolmi*). This comparison was advantageous because if the same physiological characteristics distinguishing *B. medius* from the exclusively subtidal species were also seen in *F. lapillum*, this would provide evidence that the observed characteristics of intertidal species result from selection for intertidal conditions rather than phylogeny.

Specifically, the aims of this study were: (1) to re-examine if there are differences in P_{crit} between intertidal and subtidal triplefin fishes, and (2) to identify physiological characteristics associated with P_{crit} among intertidal and subtidal triplefin fishes. To address these aims, the P_{crit} for SMR (Claireaux and Chabot 2016) was measured to establish differences in the ability to meet resting O_2 demands under hypoxia among species. Whole animal metabolic rate (SMR, $\dot{M}O_{2,max}$ and MS) was assessed to determine if a low P_{crit} is associated with a low resting O_2 demand and/or a high extractive capacity for O_2 as shown in other fishes (Richards 2011). Blood Hb concentration and gill morphometry were also measured as these may be associated with extractive capacity for O_2 and P_{crit} (Mandic et al. 2009). Finally, tissue glycogen was measured in brain, liver and white muscle, as it has been hypothesised that hypoxia-tolerant fishes have higher endogenous stores of fermentable fuels (Mandic et al. 2012; Richards 2011).

Methods

Rock pool O_2 measurements

The O_2 content of rock pools occupied by intertidal triplefin fishes was assessed to gain an understanding of the severity of hypoxia that occurs under natural conditions. Two sets of measurements were made: (1) O_2 and temperature were logged at 5 min intervals in two rock pools over a ~ 24 h period (Fig. 1A, B) and spot measurements of O_2 and temperature were taken in ten rock pools at night time low tide and then again in the same rock pools the following day at low tide (Table 2). To make the continuous measurements, O_2 loggers (D-Opto O_2 logger, Zebra-Tech Ltd, Nelson, New Zealand) were placed in rock pools for a period of ~ 24 h between the 5th and 6th of February 2019. The night and daytime spot measurements were taken on the 26th of February 2016 approximately an hour after low tide and were made at mid-depth level in the rock pools. These measurements were made with a FireSting O_2 metre and shielded temperature probe (PyroScience, Aachen, Germany). The species of fish observed in each of the ten rock pools at the time spot measurements were made was also recorded. The continuous measurements were made in rock pools located at Goat Island, Leigh, New Zealand (36°16′S, 174°47′E) and the spot measurements were made in rock pools located at Hatfields Beach, Auckland, New Zealand (36°34′S, 174°41′E). Changes in O_2 and temperature over a ~ 24 h period were also assessed at a shallow subtidal site (~ 2 m deep) at Goat Island, Leigh, New Zealand (36°18′S 174°47′E). These measurements were taken between the 5th and 6th of February 2019 using the O_2 logger described above.

Experimental animals and laboratory acclimation

Four triplefin species (*B. medius, F. lapillum, F. varium and F. malcolmi*) were collected from the wild for this study. The intertidal triplefin *B. medius* was netted from rock pools and *F. lapillum, F. varium* and *F. malcolmi* were hand netted from subtidal habitats by divers. The animals were housed in 30 L flow-through seawater tanks (air saturated, 200 µm filtered, 35 ppt salinity) at the Leigh Marine Laboratory. Fish were acclimated to normoxia and a temperature of 18 °C (± 0.5 °C) for a period of at least 4 weeks prior to experiments and were fed daily on a mixture of crushed aquaculture feed (Skretting, Australia) and pilchard. Food was withheld for a period of 48 h prior to experiments, which were performed under approval of the University of Auckland Animal Ethics Committee (AEC approval number 001441).

General respirometry methods

The mass-specific O_2 consumption rate ($\dot{M}O_2$), reported as microgram of O_2 consumed per gram of body weight per hour (mg O_2 g^{-1} h^{-1}), was determined using automated intermittent flow respirometry (Steffensen 1989). Custom-built respirometry chambers (42–210 mL) were held within a 60 L reservoir filled with filtered (1 µm) UV-sterilised seawater, which was heated or chilled to the experimental temperature (~ 18 °C) by continually pumping the seawater through a 40 L heat exchange tower containing an aluminium coil heat exchanger. The inlet of each chamber was connected to an automated Eheim compact 3000 submersible flush pump (EHEIM GmbH & Co. KG, Germany) which was switched on and off by a relay control unit (USB Power 8800 Pro, Aviosys International Inc, Taiwan) controlled by custom-coded software (Leigh Marine Laboratory). A magnetic stir bar was housed in a recess in the bottom of the respirometry chamber to ensure adequate water mixing and the O_2 concentration of water within the chamber was continuously measured using contactless sensor spots followed by FireSting O_2 metre (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 according to the equation:

$$\dot{M}O_2 = V\left(\Delta\%\frac{\text{sat}}{t}\right)a/M_B, \quad (1)$$

where V is the respirometry chamber minus fish volume, $\Delta\%\text{sat}/t$ is the change in O_2 saturation per unit time, α is the solubility coefficient of O_2 (mg O_2 $\%Sat^{-1}$ L^{-1}) in sea water (35 ppt), and M_B is the body mass of the fish in grams (Schurmann and Steffensen 1997). In all instances of $\dot{M}O_2$ assessment, the repeated measurement cycles were interspersed with 1 min periods of flushing to fully replace the water volume of the chamber. Background respiration was assessed after the fish was removed from the respirometer, but remained negligible throughout the trials. In all estimates of metabolic rate, only $\dot{M}O_2$ values with R^2 of > 0.90 for the decline in O_2 per unit of time were used.

Using the procedures above, $\dot{M}O_2$ was measured to establish the standard metabolic rate (SMR), maximum metabolic rate ($\dot{M}O_{2,max}$), aerobic metabolic scope (MS) and P_{crit} of fish (NB. SMR was measured and reported twice as part of two separate procedures. See below).

Measurement of maximum metabolic rate and aerobic metabolic scope

$\dot{M}O_{2,max}$ and MS were determined in all species at 18 °C (N = 10–11) (*B. medius*: body mass 2.32 g ± 0.37, *F. lapillum*: body mass 1.98 g ± 0.15, *F. varium*: body mass 6.1 g ± 0.85, *F. malcolmi*: body mass 6.97 g ± 0.88).

$\dot{M}O_{2,max}$ was determined following exhaustive exercise, where fish were continually chased (5 min) in a 30 L tank. An 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 best suited for obtaining $\dot{M}O_{2,max}$ in benthic species which will not continuously swim in a flume (Clark et al. 2013; Norin and Clark 2016). Following exhaustive exercise, the fish was transferred to a respirometry chamber within 30 s of the conclusion of chasing and repeating 4 min $\dot{M}O_2$ measurement cycles were initiated. $\dot{M}O_{2,max}$ was taken as the highest $\dot{M}O_2$ value recorded in any measurement cycle, which in almost all cases was obtained from the first measurement cycle following exhaustive exercise. When the metabolic rate of fish was clearly declining, the measurement period was extended to 8 min and $\dot{M}O_2$ was measured repeatedly for > 16 h. During the overnight measurement period, fish were left undisturbed in the respirometers and $\dot{M}O_2$ typically recovered to levels around SMR within ~ 6–8 h. SMR was estimated from the mean of the lowest 10% of $\dot{M}O_2$ over this time (Khan et al. 2014b; McArley et al. 2017; Norin et al. 2014). Due to the benthic habit of these triplefin species, they remain relatively inactive and perch in a stationary position on the bottom of the respirometers. This behavioural characteristic means that the lowest $\dot{M}O_2$ values recorded were probably related to periods when the fish was in an inactive state and are therefore likely to be a fair representation of SMR. MS was defined as the difference between the mass corrected $\dot{M}O_{2,max}$ and SMR (see below) for each individual.

Measurement of critical oxygen tension (P_{crit})

P_{crit} was measured in *B. medius* (body mass 3.22 g ± 0.41), *F. lapillum* (body mass 1.72 g ± 0.12), *F. varium* (body mass 6.6 g ± 0.98) and *F. malcolmi* (body mass 4.95 g ± 1.16) at 18 °C following a similar protocol to Cumming and Herbert (2016) ($N = 9–10$). In this protocol, SMR was once again measured under normoxic conditions, then a progressive hypoxic exposure was used to identify the water O_2 tension at which $\dot{M}O_2$ was no longer maintained above SMR. SMR was determined using automated intermittent flow respirometry (see respirometry methods above), where fish were left undisturbed in respirometers overnight for ~ 16 h (~ 1700–0900 hours). During overnight respirometry, $\dot{M}O_2$ was repeatedly measured over 7–9 min cycles and SMR was taken as the mean of the lowest 10% of $\dot{M}O_2$ values. $\dot{M}O_2$ measurements were then made at decreasing levels of water O_2 content (75%, 55%, 40%, 30%, 25%, 20%, 15%, 10%, and 6% of air saturation) and the required water O_2 levels were achieved by bubbling nitrogen into the seawater reservoir supplying respirometers. Three 7–9 min $\dot{M}O_2$ measurements were made at 75%, 55%, 40%, 30%, 25% and 20% of air saturation, and one 7–9 min measurement at 15%, 10% and 6% of air saturation. The progressive decline in O_2 tension to determine P_{crit} was completed in ~ 3.5 h and the time length of exposure to each O_2 level was the same for each species. To determine P_{crit}, SMR and $\dot{M}O_2$ during progressive hypoxic exposure were first mass corrected (see below), then plotted against water O_2 tension. A linear regression (forced through zero) was then established on $\dot{M}O_2$ values that fell below SMR and P_{crit} was calculated by dividing SMR by the slope of this regression line (i.e., the point where $\dot{M}O_2$ under progressive hypoxia could no longer be maintained above SMR; see Fig. 2) (as per the method of Behrens and Steffensen 2007; Cook et al. 2013; Cumming and Herbert 2016; Schurmann and Steffensen 1997).

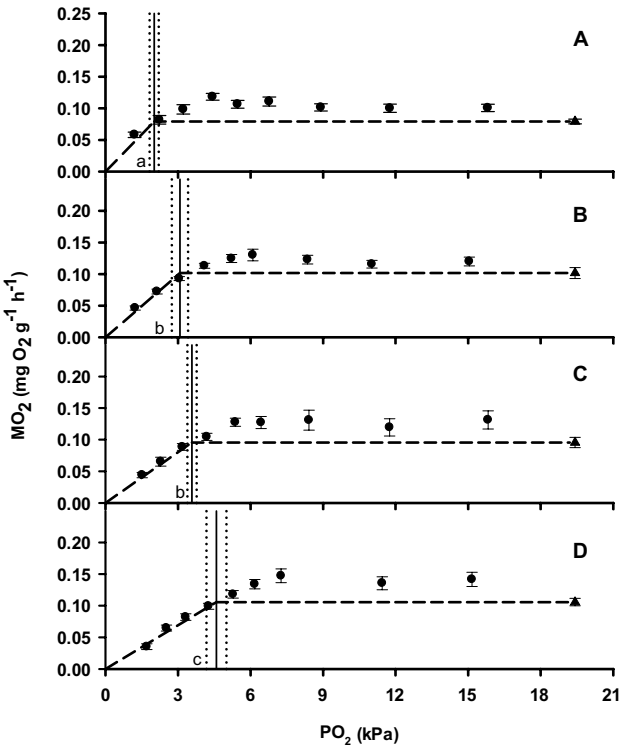

Fig. 2 Critical oxygen tension (P_{crit}) and mass-specific oxygen consumption ($\dot{M}O_2$) under progressive hypoxia exposure in *B. medius* (**A**), *F. lapillum* (**B**), *F. varium* (**C**) and *F. malcolmi* (**D**) at 18 °C. All values are mean ± SEM. and $\dot{M}O_2$ values are corrected to a body mass of 4 g ($N = 9–10$). Triangles show standard metabolic rate (SMR) under normoxia, circles show $\dot{M}O_2$ under progressive hypoxia exposure, vertical line shows P_{crit} and vertical dotted line shows P_{crit} SEM. The dashed horizontal line shows the break point where $\dot{M}O_2$ falls below SMR (i.e., P_{crit}) and is for illustrative purposes only. Different lower case letters adjacent to the vertical line representing P_{crit} show significant differences in P_{crit} between species ($P < 0.05$). There were no significant differences in SMR among species (see "Results")

Scaling of metabolic measurements

To account for body mass differences between species, the $\dot{M}O_2$ values were standardized to the mean body mass of all fish (4 g) using the formula:

$$\dot{M}O_{2(4g)} = \dot{M}O_{2(meas)} \left(\frac{w}{w_{(4g)}} \right)^{(1-A)}, \quad (2)$$

where $\dot{M}O_{2(4g)}$ is the $\dot{M}O_2$ for a fish with the standardized (corrected) new weight of 4 g, $\dot{M}O_{2(meas)}$ is the measured $\dot{M}O_2$, w is the weight of the fish, $w_{(4g)}$ is the standardized body mass of fish set to 4 g and A is the weight exponent describing the relationship between metabolic rate and body weight (Schurmann and Steffensen 1997). The mass exponent (A) used to correct $\dot{M}O_2$ to body mass was 0.8 (Clarke and Johnston 1999).

Blood haematology, tissue glycogen and gill morphometry

Haematology, tissue glycogen, and gill morphometry were assessed in *B. medius* (body mass 4.25 g ± 0.24), *F. lapillum* (body mass 2.16 g ± 0.09), *F. varium* (body mass 10.55 g ± 0.88) and *F. malcolmi* (body mass 8.74 g ± 0.81) acclimated to 18 °C for a period of 4 weeks (N = 8–10). 24 h prior to sampling, fish were transferred to individual 14 L flow-through seawater tanks so that they were not disturbed by the repeated removal of fish from the same common holding tanks. First, the fish was netted from its individual holding tank and immediately euthanized by pithing of the brain. The caudal peduncle was then severed and blood was collected by holding the tail of the fish into the base of a heparinised 0.5 mL Eppendorf tube. The brain, liver and a sample of white skeletal muscle were then immediately frozen under liquid N_2 and stored at − 80 °C. The right gill basket was removed and fixed in Bouin's solution for 48 h and then stored in 70% ethanol. Haemoglobin (Hb) concentration of fresh blood was quantified spectrophotometrically at 540 nm using modified Drabkin's reagent (Wells et al. 2007). Haematocrit (Hct) was determined in 75 mm capillary tubes spun for a period of 10 min in a haemofuge (Haemocentaur, MSE, London, UK). Tissue glycogen content was determined in thawed brain, liver and white muscle which was homogenised in ice cold 0.6 M perchloric acid. Glycogen (in glycosol units) was determined by measuring glucose content of tissue extract with and without prior incubation with amyloglucosidase as per the method of Keppler and Decker (1974). Glucose content of the tissue extract was determined using a commercially available assay kit (D-Glucose HK Assay Kit, Megazyme, Ireland).

Gill morphometry was assessed by light microscopy on intact whole gill arches (WGA) and longitudinally sectioned gill arches (SGA) from the right gill basket. All analyses of gill images were performed using Image J software (U. S. National Institutes of Health, Bethesda, MD, USA). Several gill morphometric parameters were measured including: (1) filament number (WGA), (2) filament length (WGA), (3) secondary lamellae (SL) density (WGA), (4) SL basal length (WGA), (5) SL protruding height and thickness (SGA), (6) SL bilateral surface area, and (7) mass-specific gill surface area. First, the four gill arches of the right gill basket were separated and photographed under a stereomicroscope. The number of filaments on each gill arch was counted and the length of all filaments in a single row on each arch was measured. The filament length in a single row was then multiplied by two to estimate the total length of filaments on each gill arch. To estimate the total length of gill filaments for the entire gill system (i.e., the right and left gill basket), the total length of filaments measured on each gill arch was summed and then multiplied by two. SL density was estimated by measuring the distance occupied by at least ten SL on four filaments on each gill arch. The bilateral surface area of SL (mm^2) was calculated as the area of a half ellipse based on measurements of the basal length, protruding height and thickness of SL according to the formula outlined in Matey et al. (2008). SL basal length was measured on different filaments than SL height and SL thickness due to the requirement for sectioning. As such, the mean value of each of these parameters for individual fish was used to calculate a global average of SL bilateral SA for each fish. To estimate the basal length of SL, individual filaments were removed from the third gill arch and photographed under a stereomicroscope. The width of filaments, where the base of the SL attaches, was then measured at ten evenly spaced distances along their length to approximate the basal length of the SL. To measure the thickness and protruding height of SL, the whole second gill arch was embedded in paraffin, sectioned longitudinally (5 µm) using a microtome, mounted on slides and stained with haematoxylin and eosin. The height and width of 30 SL was then measured from sections where the central venous sinuous of the filament was visible. To estimate the total surface area of the gills, the total length of gill filaments was multiplied by SL density to provide an estimate of the total number of SL in the entire gill system. The total number of SL was then multiplied by the SL bilateral surface area to produce an estimate of total gill surface area in mm^2. The total gill surface area was then standardized to body mass and is presented as mm^2 per g of body mass. To take into account the differences in the thickness of the SL between species, the maximum O_2 uptake capacity of the gills was determined according to Fick's law:

$$VO_2 = \frac{1}{t} \times K \times A \times dPO_2, \quad (3)$$

where VO_2 is the maximum O_2 uptake rate for the gills obtained by morphometric measurements, K is a diffusion constant, A is the gill area, dPO_2 is the difference between the partial pressure of O_2 on either side of the membrane and t is the thickness of the water–blood barrier (Kunzmann 1990). The thickness of the water–blood barrier (t) was taken as half the thickness of the SL and the difference in partial pressure of O_2 between seawater and blood was assumed to be 110 mmHg. K was taken as the diffusion coefficient for O_2 in water at 18 °C.

Statistics

In all statistical tests, significance was accepted at $P < 0.05$. P_{crit}, SMR, $\dot{M}O_{2,max}$, MS, gill morphometric parameters, haematological parameters and tissue glycogen were each compared between species using one-way analysis of variance (ANOVA) with Holm–Sidak post hoc comparisons. Analysis of covariance (ANCOVA) was used to determine whether there was an influence of body mass on P_{crit} and mass-specific gill surface area. Since the covariate was not found to be significant in both instances ($P > 0.05$), a standard one-way ANOVA was used to compare P_{crit} and mass-specific gill surface area among the four species. Where assumptions of normality and equality of variances were violated, data were either log transformed for the analysis or a non-parametric Kruskal–Wallis ANOVA on rank test was used. All statistical analysis was carried out using Sigma Plot 13.0 software package.

Results

P_{crit} and standard metabolic rate in hypoxia tolerance assessed fish

The four species examined showed a similar aerobic response to progressive hypoxia exposure with $\dot{M}O_2$ remaining constant and slightly elevated relative to SMR at higher PO_2, before abruptly declining and falling below SMR at lower PO_2 (Fig. 2). A difference among species in the ability to maintain $\dot{M}O_2$ above SMR under hypoxia was confirmed by significant differences in P_{crit} (ANOVA, $df = 3$, $F = 12.83$, $P < 0.001$). Post hoc comparisons showed that *B. medius* had a lower P_{crit} than all other species and *F. lapillum* and *F. varium* had a lower P_{crit} than *F. malcolmi* (Fig. 2). In the fish assessed for hypoxia tolerance (P_{crit}), the SMR of *B. medius* (0.079 mg O_2 g^{-1} $h^{-1} \pm 0.003$) was slightly lower than the SMR of *F. lapillum* (0.10 mg O_2 g^{-1} $h^{-1} \pm 0.008$), *F. varium* (0.096 mg O_2 g^{-1} $h^{-1} \pm 0.008$) and *F. malcolmi* (0.11 mg O_2 g^{-1} $h^{-1} \pm 0.005$) (Fig. 2). However, although there appeared to be a difference in SMR among species (ANOVA, $df = 3$, $F = 2.95$, $P = 0.046$), none of the pairwise post hoc comparisons were significant ($P > 0.05$).

Maximum metabolic rate and aerobic metabolic scope

In the fish assessed for $\dot{M}O_{2,max}$ and MS, there were significant differences in SMR among species (Kruskall–Wallis ANOVA, $df = 3$, $H = 11.59$, $P = 0.009$). While *B. medius* appeared to have the lowest SMR of the four species, post hoc comparisons showed the only difference in SMR was between *B. medius* and *F. varium* (Fig. 3A). There were also significant differences in both $\dot{M}O_{2,max}$ (ANOVA, $df = 3$, $F = 20.69$, $P < 0.001$) and MS (ANOVA, $df = 3$, $F = 27.39$, $P < 0.001$) between species. *B. medius* had significantly higher $\dot{M}O_{2,max}$ and MS than all other species and *F. lapillum* had significantly higher $\dot{M}O_{2,max}$ and MS than *F. varium* and *F. malcolmi* (Fig. 3B, C).

Gill morphometric parameters

There were significant differences in the number of filaments per gill arch among species (ANOVA, $df = 3$, $F = 15.31$, $P < 0.001$) with *F. lapillum* having fewer filaments than *B. medius, F. varium* and *F. malcolmi* (Table 2). The length of gill filament per unit of body mass was greater in *B. medius* and *F. lapillum* than in *F. varium* and *F. malcolmi* (ANOVA, $df = 3$, $F = 70.38$, $P < 0.001$, Table 2) and secondary lamellae density was also greater in *B. medius* and *F. lapillum* than in *F. varium* and *F. malcomi* (ANOVA, $df = 3$, $F = 123.84$, $P < 0.001$, Table 1). The basal length (Kruskall–Wallis ANOVA, $df = 3$, $H = 24.01$, $P < 0.001$), height (ANOVA, $df = 3$, $F = 32.98$, $P < 0.001$) and thickness (ANOVA, $df = 3$, $F = 46.81$, $P < 0.001$) of secondary lamellae were smaller in *B. medius* and *F. lapillum* than in *F. varium* and *F. malcolmi* (Table 2) and, as a result, the estimated bilateral surface area of the secondary lamellae was also smaller in *B. medius* and *F. lapillum* than in *F. varium* and *F. malcolmi* (Table 2). There was a significant difference in mass-specific gill surface area between species (ANOVA, $df = 3$, $F = 10.84$, $P < 0.001$) with *F. lapillum* having greater mass-specific gill surface than *B. medius, F. varium* and *F. malcolmi* (Table 2). There was some evidence that mass-specific gill surface area decreased with increasing body mass (ANCOVA: covariate, $df = 1$, $F = 3.99$, $P = 0.057$). Therefore, the high mass-specific gill surface area of *F. lapillum* could be due to the comparatively smaller body mass of this species. The maximum rate of O_2 uptake across the gills (gill VO_2) calculated according to Fick's law was significantly higher in both *B. medius* and *F. lapillum* than in *F. varium* and *F. malcolmi* (ANOVA, $df = 3$, $F = 29.19$, $P < 0.001$, Table 2).

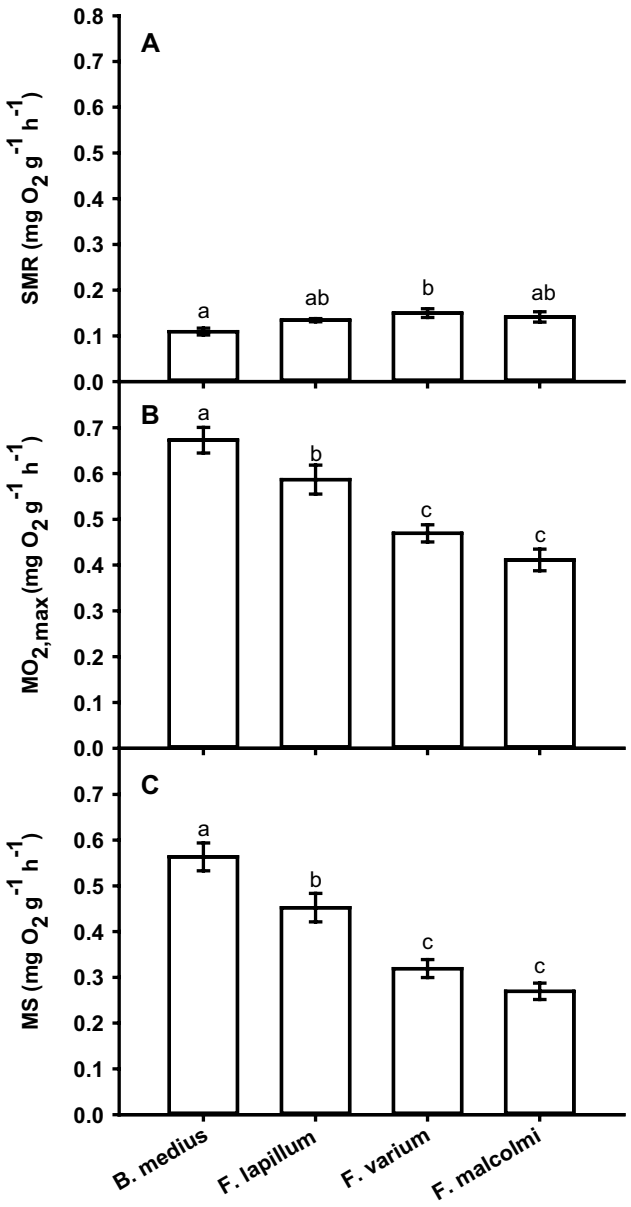

Fig. 3 **A** Standard metabolic rate (SMR), **B** maximum metabolic rate ($\dot{M}O_{2,max}$), and **C** aerobic metabolic scope (MS) in *B. medius, F. lapillum, F. varium* and *F. malcolmi* at 18 °C. All values are mean ± SEM and corrected to a body mass of 4 g ($N = 10–11$). Lower case letters not shared between bars show significant differences between species ($P < 0.05$)

Tissue glycogen stores and haematological parameters

There were differences in brain (ANOVA, $df = 3$, $F = 7.38$, $P < 0.001$) and white muscle (ANOVA, $df = 3$, $F = 17.04$, $P < 0.001$) glycogen stores among species. *B. medius* had a greater amount of glycogen in brain and white muscle than *F. lapillum, F. varium* and *F. malcolmi* and *F. lapillum* had greater white muscle glycogen than *F. malcolmi* (Table 2). There were no differences in the amount of glycogen per gram of liver tissue among species (ANOVA, $df = 3$, $F = 0.35$, $P = 0.79$), but liver size (hepatosomatic index, HSI) was also different among species (ANOVA, $df = 3$, $F = 6.85$, $P < 0.001$, Table 2). Thus, when liver glycogen was expressed per gram of body mass, there were differences among species (ANOVA, $df = 3$, $F = 3.62$, $P = 0.022$). While *B. medius* had higher liver glycogen per gram of body mass than all other species, only the post hoc comparison with *F. lapillum* was significant (Table 2). Haemoglobin concentration was different among species (ANOVA, $df = 3$, $F = 6.34$, $P = 0.002$) with *B. medius* and *F. lapillum* having a higher haemoglobin concentration than *F. malcolmi* (Table 2). There was no difference in haematocrit among species (ANOVA, $df = 3$, $F = 0.55$, $P = 0.65$).

Discussion

The pattern of P_{crit} between intertidal and subtidal triplefin species

Bellapiscis medius, which appears to be exclusively intertidal and occupies rock pools high on the shoreline (Hilton et al. 2008), has a P_{crit} which is 53%, 77% and 128% lower than *F. lapillum, F. varium,* and *F. malcolmi,* respectively. Lower P_{crit} in intertidal fish species has also been demonstrated among sculpins (Mandic et al. 2009) and the observed pattern mirrors the abrupt change in the prevalence of environmental hypoxia that exists between intertidal and subtidal habitats. *F. lapillum* was also observed in moderately hypoxic rock pools (Table 1), so the intermediate P_{crit} of this species is consistent with the balance of residing in both intertidal and relatively shallow subtidal habitats. Comparing the two exclusively subtidal species, *F. varium* had a lower P_{crit} than *F. malcolmi*, but this is unlikely to reflect a difference in exposure to hypoxia as these species overlap across depth (Table 2) and utilise almost identical habitats (Wellenreuther et al. 2007). An organism with a low P_{crit} can meet O_2 demand under more severe levels of hypoxia than can an organism with a high P_{crit} and the advantages are twofold. First, it is possible a low P_{crit} delays the initiation of anaerobic ATP production to maintain energy balance during a hypoxic event (Mandic et al. 2009; Richards 2011). Second, among intertidal and subtidal sculpins, a correlation has been demonstrated between P_{crit} and the ability to maintain an upright position (i.e., equilibrium, a proxy for survival) under severe hypoxia (Mandic et al. 2012). This suggests a lower P_{crit} plays a role in allowing intertidal fishes to survive severe hypoxia for longer periods of time than their subtidal counterparts. Although loss of equilibrium was not directly assessed in the current study, it was frequently observed in *F. varium* and *F. malcolmi* at the lowest

O_2 levels during P_{crit} determination. Furthermore, in a parallel investigation, we have observed a strong correlation between P_{crit} and time to loss of equilibrium under severe hypoxia among the four species included in the current study (Devaux et al. unpublished data). Thus, for intertidal fishes that reside in hypoxia-prone rock pools, a lower P_{crit} appears to make an important contribution to an enhanced ability to survive periods of severe hypoxia exposure.

Previous investigations by Hilton (2010) and Hilton et al. (2010) also found *B. medius* has a lower P_{crit} than subtidal triplefin species. However, P_{crit} in the current study at 18 °C was 58–157% lower for all species compared to the data of Hilton et al. at 15 °C. This likely results from the longer recovery time used in our study (16 h versus 2.5 h) and the use of intermittent flow respirometry for the determination of P_{crit} (Snyder et al. 2016). Estimates of resting $\dot{M}O_2$ in Hilton (2010) and Hilton et al. (2010) were also at least 50% higher compared to SMR in the current study and in the case of *F. varium* were 232% higher. Therefore, when hypoxia exposure was initiated only 2.5 h following entry to respirometers and closed respirometry was used, it is likely the fish under observation by Hilton et al. were still recovering from initial handling stress and that this resulted in relatively high estimates of P_{crit} because the fish were not at SMR. However, despite these discrepancies and differences in technique, all studies on triplefins to date show that intertidal species have a lower P_{crit} than their related subtidal counterparts.

Physiological characteristics associated with P_{crit} in triplefin fishes

Organisms with a high O_2 extractive capacity will be able to meet the O_2 demand of a given aerobic metabolic rate at lower O_2 tensions (Richards 2011). Therefore, among organisms which have a similar SMR, those with a high extractive capacity for O_2 should be able meet the O_2 demands of resting metabolism at lower O_2 tensions, and hence have lower P_{crit} (Cook et al. 2011). In the current study, $\dot{M}O_{2,max}$ following exhaustive exercise was assessed to provide an index of the maximum capacity of the cardiorespiratory system of each species to remove O_2 from the environment (i.e., the maximal extractive capacity for O_2). The two intertidal species *B. medius* and *F. lapillum* had higher $\dot{M}O_{2,max}$ than the exclusively subtidal species demonstrating that the cardiorespiratory system of the intertidal species has a higher capacity to take up and deliver O_2 to tissues. As there were only small differences in SMR among species, the high extractive capacity for O_2 of the intertidal species allows resting O_2 demand to be met at a lower O_2 tension and, therefore, their P_{crit} is also lower (e.g., see Cook et al. 2011). A high extractive capacity for O_2 would also lower the limiting PO_2 for other O_2 demanding activities over and above SMR (e.g., digestion, feeding, activity, etc.). This could benefit intertidal fishes as it may allow them to avoid limitation of aerobic performance for longer periods of time during hypoxia exposure (i.e., to lower O_2 tensions). While the P_{crit} of an animal is likely a function of maximal O_2 extraction capacity, it could also depend on resting O_2 demand (assessed as SMR in the current study). The intertidal specialist *B. medius* had a lower P_{crit} and higher $\dot{M}O_{2,max}$ than the other species, but there was little evidence of it also having a lower SMR (Figs. 2, 3). It was also clear that *F. lapillum, F. varium* and *F. malcolmi* had an almost identical SMR, but different P_{crit}. As a result, there was no trend between P_{crit} and SMR in the four species examined. Among sculpins, variation in routine O_2 demand (an estimate of resting metabolic rate) also explained a comparatively smaller part of the variation in P_{crit} among species than did variation in characteristics associated with extractive capacity for O_2 (HbO_2-binding affinity and mass-specific gill surface area) (Mandic et al. 2009; Richards 2011). Thus, the findings of the current study suggest that, like sculpins, the extractive capacity for O_2 plays a more important role than resting demand for O_2 in setting P_{crit} among triplefin fishes.

The observed trend of higher $\dot{M}O_{2,max}$ and lower P_{crit} in the intertidal species may be associated with a range of factors influencing the cardiorespiratory cascade (e.g., blood O_2 carrying capacity, HbO_2-binding affinity, gill morphometric parameters, and cardiac output). In the current study, the intertidal species had a higher Hb concentration than subtidal species; this could be involved as a driver of increased $\dot{M}O_{2,max}$ and lower P_{crit} because it would raise blood O_2 carrying capacity. Indeed, Cook et al. (2011) used phenylhydrazine to pharmacologically induce anaemia (i.e., lower blood O_2 carrying capacity) of a sea bream, which ultimately reduced $\dot{M}O_{2,max}$ and increased P_{crit} compared to sham controls. Secondarily, the O_2 extractive capacity of the gills could also feasibly link high $\dot{M}O_{2,max}$ and low P_{crit} together. For example, in a previous study by Mandic et al. (2009), a high mass-specific gill surface area was associated with a lower P_{crit} among 12 species of sculpins. The results of the current study provide some evidence that mass-specific gill surface area also plays a role in setting P_{crit} among triplefin fish. The mass-specific gill surface area was significantly higher in the occasionally intertidal species *F. lapillum* than in the two deeper dwelling exclusively subtidal species *F. varium* and *F. malcolmi*. This trend, however, was not observed in the intertidal specialist *B. medius* as, despite a low P_{crit}, its mass-specific gill surface area was not greater than the subtidal *Forsterygion* species. The secondary lamellae of both *B. medius* and *F. lapillum*, however, were thinner than those of the two exclusively subtidal species, suggesting a shorter diffusion distance for O_2 to reach the red blood cells during gill ventilation. Assuming that half the thickness of the secondary lamellae is representative of the blood O_2

diffusion distance across the secondary lamellae, we estimate that gill VO_2 (Table 2) was approximately double in the intertidal specialist *B. medius* and occasional intertidal occupant *F. lapillum* in comparison to the two exclusively subtidal *Forsterygion* species. Thus, the larger gill surface area and thinner secondary lamellae of the intertidal specialist *B. medius* and occasional intertidal *F. lapillum* provides a high capacity for gill O_2 flux, which likely contributes to both increased $\dot{M}O_{2,max}$ and lower P_{crit} in these species. The relationship between gill VO_2 and P_{crit} among species, however, like that for gill surface area and P_{crit}, was also not straightforward. There was no difference in gill VO_2 between *B. medius* and *F. lapillum* despite these species having different P_{crit} and there was also no difference in gill VO_2 between *F. varium* and *F. malcolmi* despite differences in P_{crit}. These discrepancies in the relationship between gill morphometric parameters and P_{crit} are likely due to the fact that P_{crit} is a complex whole animal trait influenced by a number of other physiological parameters (e.g., ventilatory capacity, cardiac output, blood oxygen binding affinity) not investigated in the current study.

Predominately, as a result of higher $\dot{M}O_{2,max}$ the intertidal specialist *B. medius* and the occasionally intertidal and shallow subtidal dwelling *F. lapillum* had higher MS (the difference between $\dot{M}O_{2,max}$ and SMR) than the deeper dwelling exclusively subtidal species *F. varium* and *F. malcolmi*. The first limitation imposed by hypoxia on aerobic capacity, which occurs at PO_2 well above P_{crit}, is a constraint on $\dot{M}O_{2,max}$ and a reduction in MS (Chabot and Claireaux 2008; Claireaux and Lagardère 1999; Claireaux and Chabot 2016; Claireaux et al. 2000; Cook et al. 2011; Farrell and Richards 2009; Lefrançois and Claireaux 2003). Exposure to increased temperature, as occurs for intertidal fish during the day in rock pools (e.g., Fig. 1), also limits MS because increased resting O_2 demand reduces the difference between SMR and $\dot{M}O_{2,max}$ (Pörtner and Knust 2007; Schulte 2015). As aerobic activities (e.g., growth, feeding, and activity) must occur within the bounds of MS, it is thought that MS-limiting environmental conditions can cause energy budgeting conflicts which lead to a loss of whole organism performance (e.g., reduced growth in Atlantic cod under hypoxia) (Chabot and Claireaux 2008). Potentially, a high MS provides a reserve of aerobic capacity which intertidal fishes can utilise to avoid severe constraints on performance under exposure to hypoxia or high temperature in rock pools. Further studies examining the effects of hypoxia on other aerobic activities (e.g., specific dynamic action, which is probably forced to operate within the bounds of MS) would therefore provide useful insight into whether rock pool fishes utilise high MS to their advantage.

The pattern of MS observed among species in the current study could also result from different regimes of wave exposure between intertidal, shallow subtidal and deeper subtidal habitats. Hickey and Clements (2003) determined that intertidal triplefins are subjected to substantially higher water velocities than those species occupying subtidal habitats, and that water velocities resulting from wave action decline progressively with increasing habitat depth. Thus, the higher MS of the specialist intertidal species *B. medius* and the occasionally intertidal and shallow subtidal dwelling *F. lapillum* could reflect an increased requirement for activity (e.g., station holding) in the high water velocities of intertidal and shallow habitats. The comparatively lower MS of *F. varium* and *F. malcolmi* may reflect a lower demand for activity as the water velocities resulting from wave action will not be as high in the deeper subtidal habitats occupied by these species.

Although a low P_{crit} is beneficial for fish exposed to hypoxia, it does not preclude exposure to O_2 tensions capable of threatening survival. Indeed, intertidal fish are more at risk of exposure to O_2 tensions less than P_{crit} than their subtidal counterparts, because environmental hypoxia does not regularly occur in the habitat of the latter. At O_2 tensions below P_{crit}, organisms rely at least partly on O_2 independent ATP production to maintain energy balance. This is evidenced by the accumulation of lactate in the tissues and plasma of fish exposed to O_2 tensions below P_{crit} (Herbert and Steffensen 2005; Scott et al. 2008; Speers-Roesch et al. 2013). Thus, fish species that reside in hypoxia-prone habitats should have large stores of endogenous fermentable fuels such as glycogen (Richards 2011). The intertidal specialist *B. medius* maintained a higher resting concentration of glycogen in white skeletal muscle and brain tissue than the occasional intertidal occupant *F. lapillum*, and the two exclusively subtidal *Forstyregion* species. Although there was no difference in liver glycogen concentration between species, *B. medius* did have a larger liver relative to body mass. Thus, there was some evidence of a trend for increased liver glycogen stores relative to body mass in *B. medius*, which fits with the theory outlined above that intertidal fishes may store higher levels of endogenous fermentable fuels to power anaerobic metabolism under hypoxia exposure. Higher tissue glycogen stores in intertidal fishes could also be a response to a more variable food supply, as a limitation in food availability may be a common occurrence in rock pool habitats (Silberschneider and Booth 2001).

A limitation of the current study is that the intertidal specialist *B. medius* is placed in a different genus than the subtidal species examined. Thus, we cannot rule out the possibility that the observed differences in physiological traits between *B. medius* and the subtidal species are a result of phylogeny rather than adaptation to rock pool conditions. It is noteworthy, however, that *F. lapillum*, a species which also occurs in the intertidal had a lower P_{crit}, and higher $\dot{M}O_{2,max}$, MS, blood Hb concentration, and gill VO_2 than exclusively subtidal species *F. varium*

and *F. malcolmi*. Thus, the physiological characteristics of *B. medius* that appear suited to a rock pool existence also differentiated *F. lapillum* from two closely related subtidal species within its own genus. Future studies that include a greater number of species and take phylogeny into account would be beneficial in further elucidating the adaptive value of the physiological traits examined in the current study.

Conclusions

The present investigation found that the specialist intertidal triplefin species *B. medius* possesses a lower P_{crit} than the occasional rock pool inhabitant *F. lapillum,* and the two exclusively subtidal species *F. varium* and *F. malcolmi*. *F. lapillum* also had a lower P_{crit} than two closely related exclusively subtidal species (*F. varium* and *F. malcolmi*). *B. medius* also had a higher $\dot{M}O_{2,max}$ than all three *Forsterygion* species and *F. lapillum* had a higher $\dot{M}O_{2,max}$ than *F. varium* and *F. malcolmi.* These findings demonstrate that an enhanced capacity of the cardiorespiratory system to extract O_2 from water is likely to be an important determinant of P_{crit} in triplefin species which inhabit intertidal rock pools. There was, however, virtually no association between SMR and P_{crit} among the species examined. This finding suggests that among triplefin fishes, a high extractive capacity for O_2 is a comparatively more important determinant of P_{crit} than a low resting demand for O_2. Overall, triplefin species which can extract more O_2 from their environment have a lower P_{crit} and therefore, have the ability to meet aerobic metabolic demands at lower O_2 tensions. A lower P_{crit} is advantageous for an exclusively intertidal species such as *B. medius*, because environmental hypoxia is a routine occurrence in its rock pool habitat. High $\dot{M}O_{2,max}$ also endows intertidal species with a high MS, and therefore a greater capacity to perform aerobic activities over and above maintenance requirements. Whether high MS in intertidal fish can be utilised to mitigate constraints on aerobic performance under hypoxia and high temperature, would be an interesting avenue for future research.

Acknowledgements T.J.M. would like to acknowledge support from the University of Auckland Scholarship Office for doctoral funding. Supplementary funds were also provided by a Marsden Grant awarded to A.J.R.H and N.A.H. (UOA1407: How to avoid brain damage during oxygen deprivation? Intertidal fish provide a unique test model). Technical staff Peter Browne and Errol Murray are acknowledged for their assistance with design and fabricating the respirometry setups and John Atkins is acknowledged for design of the respirometry software. Dr. Nicholas Shears and Dr. Caitlin Blain are thanked for allowing us to use of their oxygen and temperature loggers. We also thank three anonymous reviewers for their effort in providing comments and critiques which were helpful in revising an original version of this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (University of Auckland AEC approval number 001441).

Data availability The data generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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