

Physiology underpins habitat partitioning in a sympatric sister-species pair of intertidal fishes

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Summary

1. Integrative ecophysiological studies of closely related taxa are required to explore the causative factors that determine species distributions, and can offer insight into the selective forces that may have driven evolutionary divergence.
2. We examined the distribution, abundance, size and microhabitat associations of two sympatric sister-species of intertidal fishes *Bellapiscis medius* and *B. lesleyae* (Family Tripterygiidae) that occupy intertidal rockpools along the coastline of New Zealand, to examine the ecological factors that determine habitat choice in these species.
3. We also measured rockpool water temperatures and examined rates of oxygen consumption, ventilation frequency and hypoxia tolerance in each species at ecologically relevant temperatures to assess whether differences in respiratory physiology are associated with differences in vertical distribution.
4. The results showed clear interspecific differences in vertical habitat partitioning, with *B. medius* occupying rockpools higher on the shore than its congener. The two species also differed significantly in adult body size and aggregation size, with *B. medius* being significantly larger and occurring in lower numbers per rockpool than *B. lesleyae*.
5. *Bellapiscis medius* in upper-shore rockpools are exposed to higher extremes of temperature and presumably dissolved oxygen than *B. lesleyae*. Despite having similar rates of weight-specific oxygen consumption, *B. medius* showed a greater tolerance of hypoxia (lower critical oxygen tension) than *B. lesleyae* at both high (25 °C) and low (15 °C) temperatures, as well as a lower sensitivity to acute temperature change as indicated by differences in ventilation frequency. We thus suggest that the greater physiological tolerance exhibited by *B. medius* is likely to be an important factor enabling this species to exploit the higher shore environment.
6. Our integrative approach enabled us to demonstrate a clear link between ecological divergence and physiological performance in these intertidal fishes. Differing physiological tolerance is probably an important factor facilitating co-existence of these sister-species in sympatry, and is likely to have been a factor in their speciation.

Key-words: ecological divergence, habitat choice, hypoxia, respiratory physiology, temperature, Tripterygiidae

Introduction

It is now firmly established that physiology plays a crucial role in determining species ranges (Somero 2002; Chown, Gaston & Robinson 2004). Not only does physiology play a direct role through variation in tolerance of abiotic factors such as temperature (Somero 2002; Stillman 2002), but organismal

physiology can also be an important modifier of intra- and interspecific biotic interactions such as competition (Wetthey 1984; Menge, Olson & Dahlhoff 2002; Seebacher & Wilson 2006) and predation (Sanford 1999). Integrative studies are therefore necessary to examine the physiological and ecological factors that may influence distributional patterns.

The marine intertidal zone provides an excellent study system for integrating physiology and ecology to explain species distribution along the gradient from low to high tide level. Species occupying intertidal rockpools frequently experience rapid and extreme fluctuations of temperature,

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salinity, pH, oxygen (O₂) and carbon dioxide relative to the subtidal (Daniel & Boyden 1975; Truchot & Duhamel Jouve 1980; Morris & Taylor 1983; Bridges *et al.* 1984), with the height of the rockpool above low tide determining the duration of emersion and hence affecting the magnitude and duration of these fluctuations (Daniel & Boyden 1975; Metaxas & Scheibling 1993). Animals inhabiting intertidal rockpools must therefore have behavioural and/or physiological mechanisms which allow them to survive these large and rapid environmental changes (Gibson 1982; Horn, Martin & Chotkowski 1999).

Rockpools also provide an ideal opportunity to study habitat partitioning in co-occurring species, as rockpools are discrete spatial units with high numbers of quantifiable habitat variables (Metaxas & Scheibling 1993; Davis 2000). Mechanisms of resource partitioning in the rocky intertidal include differences in daily activity cycles (Davis 2001), seasonal cycles (Berger & Mayr 1992; Davis 2000), shelter sites (Faria & Almada 2001; Silberschneider & Booth 2001), dietary specialization (Szabo 2002) and vertical zonation (Raffaelli & Hawkins 1996; Zander, Nieder & Martin 1999). Out of these factors, vertical habitat partitioning has been found to be common among intertidal fishes (Zander *et al.* 1999; Davis 2000), and temperature and O₂ availability are thought to exert a profound influence on vertical distribution patterns through differences in physiological tolerance (Gibson 1982; Somero 2002). However, variation in species tolerances may interact with biological factors such as competition or predation to determine the realized distribution of a species (Connell 1961; Zander *et al.* 1999; Tomanek & Helmuth 2002).

The ideal candidates for comparative physiological and ecological studies are closely related taxa, where phylogenetic relationships are known (Huey 1987; Harvey & Pagel 1991). The New Zealand species of triplefin fishes (Family Tripterygiidae) form an excellent model system for comparative study as most species are closely related (Hickey & Clements 2005) and distributed sympatrically throughout New Zealand's coastal waters (Fricke 1994). The subtidal species exhibit strong (Wellenreuther, Barrett & Clements 2007) and consistent habitat partitioning (Wellenreuther, Syms & Clements 2008), and display high site fidelity (Clements 2003). These species-specific habitat associations appear to be the result of active choice by larvae (Wellenreuther & Clements 2008), and habitat differences contribute significantly to the isolation between subtidal species during the reproductive season (Wellenreuther & Clements 2007). *Bellapiscis medius* is the only New Zealand triplefin species which is not recorded in subtidal surveys (Wellenreuther *et al.* 2007), and thus appears to be exclusively intertidal. However, it co-occurs with its sister-species *B. lesleyae* in rockpools at low tide throughout New Zealand including the Three Kings Islands, Stewart Island and the Chatham Islands wherever there is suitable habitat (Fricke 1994). Although they are morphologically very similar, the species differ in maximum length, head shape, posterior extension of the lateral line, and colour pattern and have been recognized as distinct species since

1987 (Hardy 1987). The fine-scale distribution patterns and abundance of this species pair within the intertidal zone are poorly understood, and thus it remains unclear whether and how the species partition resources within the intertidal habitat. Both species are micro-carnivores which feed on small benthic invertebrates (Feary 2001). This lack of dietary specialization indicates that other factors, such as micro-habitat, could be important in resource partitioning, and the presence of *B. lesleyae* but not *B. medius* in subtidal transect surveys suggests that, despite their co-occurrence in intertidal rockpools, they may differ in habitat use (Wellenreuther *et al.* 2007).

We examined the abundance, size and distribution of adult *B. medius* and *B. lesleyae* in intertidal rockpools to investigate if and how these species partition the intertidal habitat. We examined rockpool depth, volume, surface area, height above chart datum (ACD), exposure and proportional abundance of different substrate types together with abundance counts and size measurements of individuals to identify which ecological factors were good predictors of rockpool occupancy for each species. We then measured rockpool water temperatures experienced by both species, and investigated their respiratory capacity, including resting rates of O₂ consumption, ventilation frequency and critical O₂ concentration, at ecologically relevant temperatures and in relation to acute temperature change and gradual hypoxia. These physiological experiments aimed to determine whether interspecific differences in tolerance of fluctuations in temperature and O₂ availability are linked to ecological factors that determine rockpool occupancy.

Materials and methods

ECOLOGICAL DATA COLLECTION: FIELD SURVEYS

Habitat surveys were undertaken at eight locations in the North Island of New Zealand: Taemaro Bay (173°34'5' E, 34°56'5' S), Matheson's Bay (174°48' E, 36°18' S), Takapuna Beach (174°46'5' E, 36°47' S), Waiheke Island (175°1'5' E, 36°46'5' S), Fletcher Bay (175°23'5' E, 36°28'5' S), Makara Beach (174°43' E, 41°13' S), Island Bay (174°46'5' E, 41°21'5' S) and Princess Bay (174°47'5' E, 41°21'5' S) (Fig. 1). Surveys were conducted between May 2003 and June 2004 during low tides of ≤ 0.6 m ACD. A total of 109 intertidal rockpools contained *B. medius* and/or *B. lesleyae*. Clove oil anaesthetic was added to the rockpool water to increase efficiency of recovery of these small and cryptic species. Fishes were subsequently removed by hand or dip net and placed in fresh seawater to recover. Fishes were visually identified to species according to the differences in colour pattern, posterior extension of the lateral line scales and relative depth of the caudal peduncle (Hardy 1987). After identification all fishes were allowed to fully recover and were released into the sea.

To estimate the size distribution of *B. medius* and *B. lesleyae* in the intertidal, a sample of both species was collected from rockpools at Matheson's Bay, Takapuna Beach and Waiheke Island during the reproductive season (June–September) and measured to the nearest millimetre (total length, L_T). Sampling during the reproductive season ensured that all fishes were adults, as the triplefin recruitment season occurs approximately from October to March (Wellenreuther & Clements 2007).

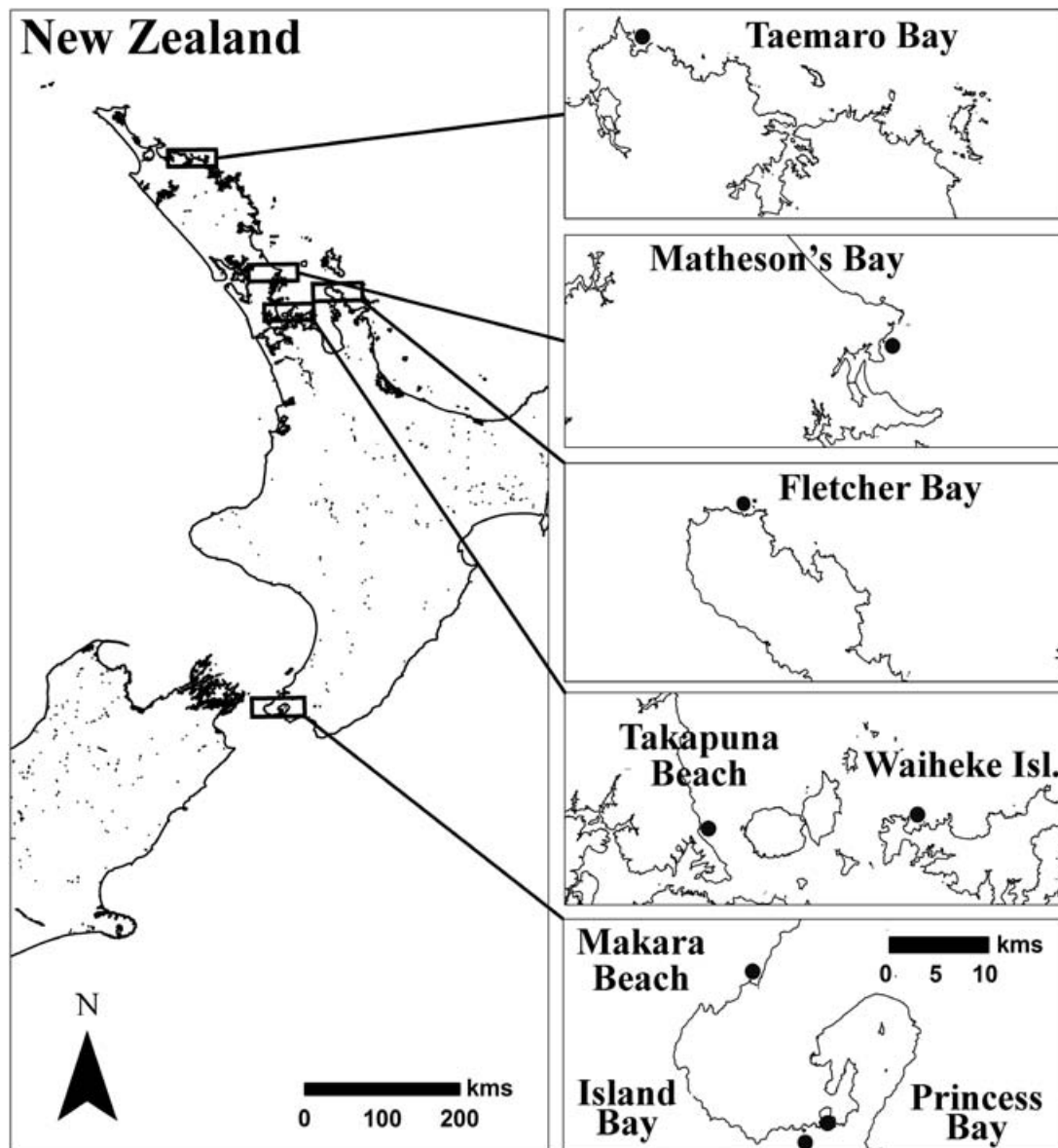


Fig. 1. Location of the eight survey sites in the North Island of New Zealand; Taemaro Bay, Matheson's Bay, Fletcher Bay, Takapuna Beach, Waiheke Island, Makara Beach, Island Bay, and Princess Bay (for coordinates see Methods). For physiological experiments fish of both species were pooled from the Matheson's Bay and Takapuna sites. Rockpool temperature measurements were carried out at the Matheson's Bay site.

For each rockpool, the location was georeferenced using a handheld Garmin® 12 global positioning system (GPS) unit (accuracy ± 15 m). This information was used to calculate a physically derived exposure index (Thomas 1986) for each rockpool using the program Fetch Effect Analysis VI (available from the author: cr_pickard@hotmail.com). The height ACD was recorded for each pool using a surveyor's dumpy level (Leica-Geosystems, Heerbrugg, Switzerland) and was corrected for barometric pressure using local meteorological data. The length, width and depth of each rockpool were measured to the nearest centimetre. Pool surface area (SA) was estimated by multiplying mean length by mean width, and pool volume was estimated by multiplying SA by mean depth. The substratum type and amount of algal cover were estimated visually as percentage cover, always by the same observer (M. Wellenreuther). Substratum was defined as rock (rocks > 7 cm), cobbles (rocks 4–7 cm), gravel (rocks < 4 cm) or sand.

To estimate the actual water temperatures experienced by fish inhabiting these rockpools, water temperature measurements were made at the Matheson's Bay site using three 'StowAway TidbiT®' temperature data loggers (Onset Computer Corp., Bourne, MA). All rockpools at the site were examined and one data logger was placed in the highest rockpool containing an adult *B. medius* (1.38 m ACD), a second data logger in the highest pool containing *B. lesleyae* (0.19 m ACD), and the third in the highest rockpool at the site (2.33 m ACD). All data loggers recorded rockpool temperatures at 10 min intervals and were placed at mid-depth of the pool, attached to the rock with a small expansion bolt, and left for 1 month before data recovery. Temperature logging was done in August (winter) 2004, November (spring) 2004 and February (summer) 2006. Local sea surface temperature (SST) data since 1967 indicate that lowest SSTs occur in August and highest in February/March (Leigh Marine Laboratory Climate Data Archives).

PHYSIOLOGICAL DATA COLLECTION: RESPIROMETRY

Adults of both *B. medius* and *B. lesleyae* were caught from rockpools at Matheson's Bay and Takapuna Beach (see Fig. 1) during low tide by hand-net without using clove oil. Fishes were acclimated in 30 L tanks in fully aerated recirculating seawater at either 15 or 20 °C for at least 4 weeks before experimentation. Fishes were fed to satiation daily on a mixture of krill, mysis shrimp, mussel, bloodworms and *Artemia*. Individuals were not fed for 1 day before experimentation to remove specific dynamic action.

Closed-cell respirometry was performed in cylindrical Perspex® respirometry chambers measuring 50 × 47 mm, 68 mL or 70 × 66 mm, 212 mL, depending on fish size, so that movement was not restricted. Both species are demersal and after a short initial period of activity remained still on the bottom of the chamber. The chamber contained a Clark-type O₂ electrode embedded in the ceiling and a perforated Perspex® floor to house the fish above a small magnetic stirrer. The entire chamber was submerged in a temperature-controlled aquarium kept at 100% O₂ saturation. Dissolved O₂ concentration (DO) in the respirometry chamber was measured as percentage of air saturation using a 781 Oxygen Meter (Strathkelvin Instruments Ltd., Scotland), and recorded and analysed using 949 Oxygen System software (Strathkelvin Instruments Ltd., Scotland). DO in the water was converted to mg O₂ L⁻¹ at each temperature using O₂ solubility coefficients for each temperature at the appropriate salinity obtained from published tables.

To measure resting rates of O₂ consumption (VO₂), individuals of each species were placed in the respirometry chamber under flow-through conditions in 100% O₂ saturated seawater at their relevant acclimation temperature (15 or 20 °C). The water temperature was then raised or lowered over a period of 2 h to the desired measurement temperature of 10, 15, 20 or 25 °C. The acclimation and measurement temperatures and the acute temperature change were selected to mimic natural conditions observed in the rockpools where fishes were collected. The mean annual SST in the Matheson's Bay area is 17 °C (Leigh Marine Laboratory Climate Data Archives), and temperatures ranging from 10 to 27 °C were measured in a Matheson's Bay rockpool containing *B. medius* (see results). Fish acclimated at 15 °C were measured at the full range of measurement temperatures (10–25 °C), however fish acclimated at 20 °C were only measured at the highest temperature (25 °C). Preliminary experiments were also attempted at 30 °C but this temperature appeared to be beyond the tolerance limits of both species, and therefore we did not pursue the experiment further. For all other temperatures the experimental procedures were as follows. Once the desired measurement temperature was reached, fishes were left to settle for 30 min at the new temperature before the experiment started. Initial trials showed that 30 min was sufficient time for fish to calm and achieve a constant ventilation rate and VO₂. The chamber was then sealed and the fishes were left to consume 50% of the available O₂. The fishes were then removed from the chamber, blotted dry and weighed by adding to a known weight of fully oxygenated 15 °C water. Finally, the fishes were returned to the aquaria to recover. Blank measurements were made to account for microbial respiration in the seawater but this was consistently negligible. Fishes were sometimes agitated by initial closing of the chamber, leading to a slightly elevated VO₂ for the initial period of the experiment. The initial data were thus discarded and VO₂ calculated as the (consistently linear) slope of the declining O₂ trace between 80% and 60% O₂ saturation.

To measure critical oxygen concentration (O_{2 crit}; defined as the concentration of O₂ below which the fish can no longer maintain a constant VO₂ independent of the ambient DO, that is, the point of

change from oxyregulation to oxyconformation), experiments were carried out as above, however fishes were left in the closed chamber and the decline in O₂ was monitored until the fishes began to exhibit 'escape behaviour' which consisted of fishes darting rapidly around the chamber. At the first sign of escape behaviour fishes were immediately removed from the chamber, quickly weighed as above, and placed into fully oxygenated 15 °C seawater and allowed to recover. The O_{2 crit} experiment was conducted at 15 and 25 °C only. The O_{2 crit} was calculated from each trace of declining O₂ saturation by first dividing the trace into intervals of 5% O₂ saturation and calculating the VO₂ for each interval. The O_{2 crit} was then calculated as the intersect of the regression lines of VO₂ regulation and VO₂ conformation using segmented least-squares linear regression performed using the software SEGREG (Oosterbaan 1994).

During the hypoxia tolerance experiments, opercular ventilation frequency was recorded by the same observer (Z. Hilton) counting the frequency of opercular movements for periods of 15 s and the measurement expressed as opercular beats per minute (BPM). This was repeated at every 10% interval in DO from 100% until termination of the experiment. The air saturation was then converted to mg O₂ L⁻¹ to account for differences in O₂ solubility with temperature.

STATISTICAL ANALYSES

Species presence and absence data for each rockpool were analysed with a Classification and Regression Tree analysis (CART; Breiman *et al.* 1993) to investigate which rockpool habitat variables discriminated best between the species. CART analysis uses a statistical approach to find specific variable values which will produce subsets of data that best distinguish between species presence and absence (De'Ath & Fabricius 2000). In CART analysis the data are split repeatedly into subsets to produce a tree consisting of a hierarchy of nodes, where each successive split represents the next most important variable. For the current analysis the fully constructed tree was subsequently constrained to remove redundant nodes using a holdout sample cross-validation. Predictor variables used in the analysis were rock, cobbles, gravel, sand, algae, pool surface area, pool volume, rockpool height ACD and exposure.

Simple linear regressions were used to further examine the relationships between intertidal height ACD and the other rockpool characteristics. To analyse the abundance of each species in rockpools, independent *t*-tests were performed using the Welch test to account for inequality of variance between the two species (Zimmermann 2004). To test for intraspecific trends towards increasing or decreasing body size in relation to vertical distribution, the body size of each fish was correlated with rockpool height ACD using simple linear regression. All statistical tests were performed in STATISTICA V 6.1. (StatSoft, Inc., USA), with the exception of the Welch test which was carried out in SPSS V 12.01 (Lead Technologies Inc., Chicago).

Both *Bellapiscis* species exhibited allometric scaling of VO₂ and O_{2 crit} with body size, with scaling coefficients being both temperature- and species-specific. For comparative purposes we therefore normalized all measurements to 1 g body weight using the exponents derived from least-squares linear regression of log/log transformed VO₂ or O_{2 crit} and body size for each species at each temperature. Normalized VO₂ was expressed as mg O₂ consumed per hour per gram ± standard error of the mean (SE). Ventilation frequency did not show allometric scaling within species, and thus was expressed simply as mean opercular beats per minute (BPM) ± SE.

For both VO₂ and O_{2 crit} data, a two-factor analysis of variance (factors: species, measurement temperature) was performed with Type III Sum of squares using 'R' V 2.4.1 (The R Foundation for

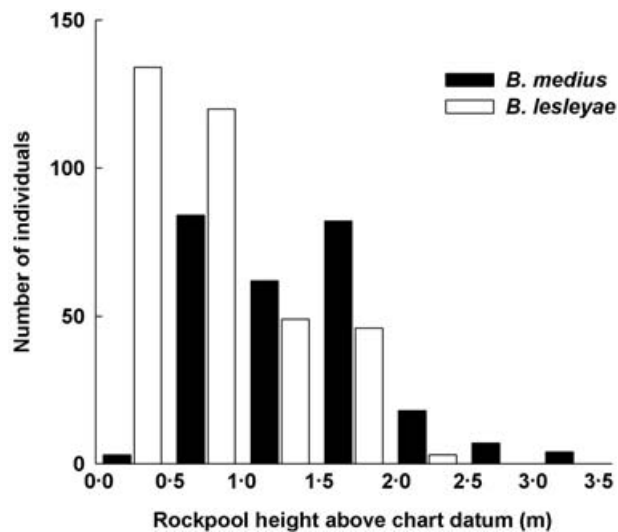


Fig. 2. Vertical distribution and abundance of *B. medius* (black; $n = 260$) and *B. lesleyae* (white; $n = 352$) from 109 surveyed rockpools.

Statistical Computing, Vienna, Austria) followed by Tukey's pairwise comparisons using SAS V 9.1 (SAS Institute Inc., Cary, NC). Assumptions of ANOVA were tested using residual analysis. For the VO_2 analysis data were log transformed. For comparison between acclimation temperatures, the above analysis was repeated (factors: species, acclimation temperature) and data were log transformed. For all analyses $\alpha = 0.05$.

Results

HABITAT PARTITIONING IN *BELLAPISCIS MEDIUS* AND *BELLAPISCIS LESLEYAE*

The two species differed in habitat use, most notably in their vertical distribution among rockpools. *Bellapiscis medius* was found in relatively high-shore rockpools with a mean height of 1.39 m ACD (range 0.31–3.39 m ACD, Fig. 2), while *B. lesleyae* was found predominantly in low intertidal rockpools with a mean height of 0.71 m ACD (range 0–2.20 m ACD, Fig. 2). The relative proportion of each species changed markedly with increasing shore height, with the relative abundance of *B. medius* increasing from 2% in rockpools at 0–0.5 m ACD to 100% in those above 2.5 m ACD (Fig. 2).

Tests of correlation between rockpool substratum variables and rockpool height ACD showed that percent cover of bare rock had a significant positive correlation with height ACD ($r^2 = 0.14$, $P < 0.001$) and percent cover of gravel had a significant negative correlation with height ACD ($r^2 = 0.09$, $P < 0.01$). The magnitude of the correlation coefficients (r^2) for both regressions were, however, relatively low. No significant correlations were found between height and the variables cobble, sand, algal cover, rockpool surface area, rockpool volume or exposure.

The CART analysis was used to identify which of the ecological variables (vertical height and substratum variables) had the most predictive power in determining the presence/

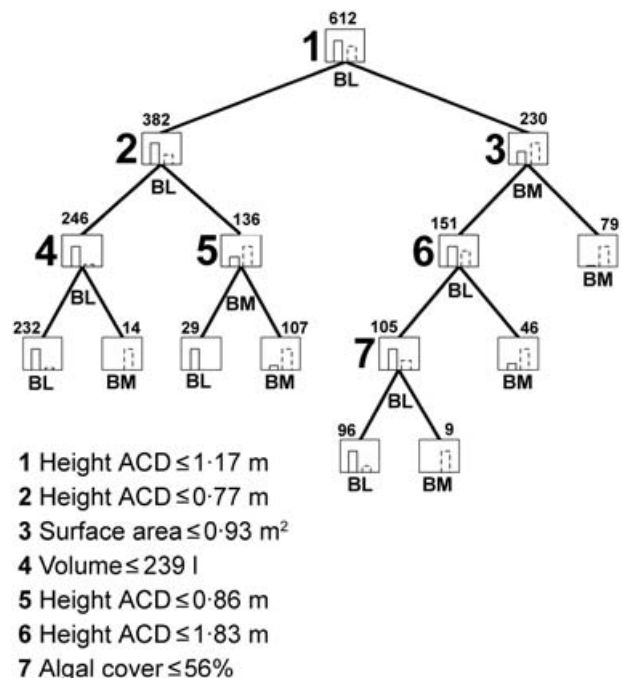


Fig. 3. Results of CART analysis, a type of decision tree using habitat variables as predictors of species membership. Decisions are made iteratively. The *small numeral* above each node depicts sample size. The *large numeral* depicts the significant classifying variables or 'decisions' 1–7. For each decision; if yes, follow branch to the left, if no, follow branch to the right. Each histogram depicts relative number of *B. lesleyae* (solid line) and *B. medius* (dotted line) correctly assigned by the previous decision. Total number of individuals $n = 612$. Dependent variables were species; *B. medius* (BM; $n = 260$) or *B. lesleyae* (BL; $n = 352$). Predictor variables were rockpool surface area (m²), rockpool volume (L), rockpool height above chart datum (ACD; m), exposure, and rockpool substratum classified as percentage cover of rock, cobbles, gravel, sand, or algae.

absence of the two species. Species membership of 89% of individuals could be accurately predicted based on just four rockpool habitat variables: height ACD, surface area, volume and percentage cover of algae (Fig. 3). From these four variables, rockpool height produced the first split, and three of the remaining six splits in the tree. The successive splits due to this single variable were produced because both species overlapped in mid-shore rockpools (Fig. 2). Thus rockpool height was clearly the most important predictor of species occupancy, confirming the observed vertical habitat partitioning between species. Within the higher shore rockpools (> 1.17 m ACD), surface area (SA) was also found to be a good classifier, splitting the data into pools of larger SA (≥ 0.93 m²) containing almost exclusively *B. medius*, and smaller SA (≤ 0.93 m²) containing both species. Height ACD further split these occupants of the lower SA rockpools with *B. medius* almost exclusively occupying the highest pools (> 1.83 m ACD). For the remaining individuals occupying high shore (1.17–1.83 m ACD) lower SA rockpools, algal cover split the tree into pools with a density of algae $\leq 56\%$ containing mostly *B. lesleyae* versus those with a density of algae $\geq 56\%$

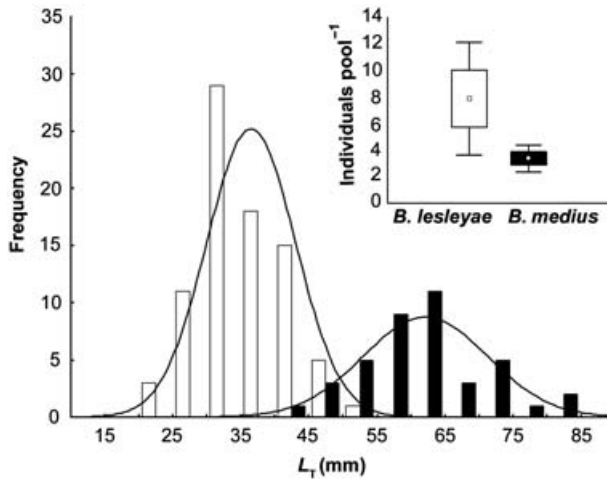


Fig. 4. Size frequency was measured as total length (L_T ; mm) of adult *B. medius* (black; $n = 54$) and *B. lesleyae* (white; $n = 94$). Inset shows number of individuals found per rockpool. The small square denotes the mean, the large rectangle denotes mean ± 1 SD, and error bars denote mean ± 1.96 SD. The number of surveyed rockpools that contained ≥ 1 individual was 47 and 65 for *B. lesleyae* and *B. medius*, respectively.

containing *B. medius*. Within the lower-shore pools (< 1.17 m ACD), height ACD again split the species twice, with *B. medius* always occupying the higher rockpools. Of those fishes occupying the lowest pools (< 0.77 m ACD) rockpool volume was also a significant classification variable with the small number of *B. medius* present at these low shore elevations ($n = 14$) occurring in the very large rockpools (≥ 239.1). In summary, *B. medius* is more abundant in higher shore rockpools often with a larger surface area and higher algal cover, while *B. lesleyae* occupies rockpools lower on the shore.

BODY AND AGGREGATION SIZE

The species differed in adult body size, and overlap was minimal (Fig. 4). The mean body size was 3.64 ± 0.65 cm for *B. lesleyae* and 6.19 ± 0.14 cm for *B. medius*. Size ranges were 4.0–8.5 cm L_T and 2.1–5.0 cm L_T for *B. medius* and *B. lesleyae*, respectively. Although the species' size ranges were large, no intraspecific effect of body size on the position in the intertidal was detected for either species (*B. lesleyae*: $r = -0.07$; $P = 0.51$; *B. medius*: $r = 0.19$, $P = 0.13$), suggesting that small and large individuals are evenly distributed in the species' habitat space.

The two species differed significantly in the mean number of individuals per rockpool ($t_{54,42} = 2.53$, $P < 0.01$, Welch $P = 0.01$, Fig. 4). Aggregations of more than nine *B. medius* individuals were only found in three instances, and all in rockpools < 1.10 m ACD. Similarly, all of the very large aggregations of *B. lesleyae* (> 25 individuals) occurred in rockpools < 0.2 m ACD. There was a significant negative correlation of aggregation size with rockpool height for *B. lesleyae* ($r = -0.28$; $P = 0.02$) but not for *B. medius*: ($r = -0.01$; $P = 0.94$). The size of the aggregation was significantly

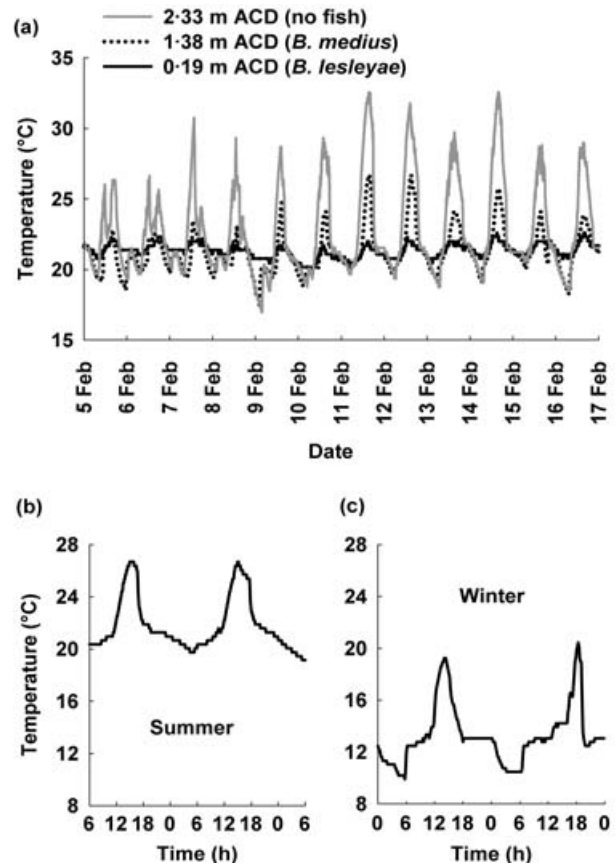


Fig. 5. (a) Rockpool water temperatures at the Matheson's Bay study site ($174^{\circ}48' E$, $36^{\circ}18' S$) during February 2006 (late summer). Three data loggers simultaneously measured temperature in the highest rockpool on the shore (2.33 m ACD; solid grey line), the highest rockpool which was found to contain an adult *B. medius* (1.38 m ACD; dotted line), and the highest rockpool found to contain an individual *B. lesleyae* (0.19 m ACD; solid black line). (b) and (c) Rockpool water temperatures measured in the 1.38 m ACD rockpool (containing *B. medius*) over a 48 h period during (b) summer (11–12 Feb 2006) and (c) winter (3–4 Aug 2004).

related to both pool volume and surface area in *B. medius* (pool volume: $r = 0.51$, $P < 0.001$; surface area: $r = 0.48$, $P < 0.001$) but not in *B. lesleyae* (pool volume: $r = -0.1$, $P = 0.56$; surface area: $r = -0.04$, $P = 0.83$).

OBSERVED ROCKPOOL TEMPERATURES

Temperature fluctuations recorded in the highest rockpool at Matheson's Bay were of up to $13^{\circ}C$ on a diurnal cycle in summer, with temperatures reaching over $32^{\circ}C$ (Fig. 5a). In contrast, the highest rockpool containing an individual of *B. medius* experienced fluctuations of up to $7^{\circ}C$ and a maximum of $26^{\circ}C$, and the highest pool containing *B. lesleyae* fluctuated by just $2^{\circ}C$ with a maximum of $22^{\circ}C$ over the same time-period (Fig. 5a). Temperature ranges recorded in the highest rockpool occupied by *B. medius* varied over the seasons from 9 – $20^{\circ}C$ in winter (August), 14 – $26^{\circ}C$ in spring (November) and 17 – $27^{\circ}C$ in summer (February). The low rockpool

Table 1. Values from the experiment measuring oxygen consumption versus temperature in *B. medius* and *B. lesleyae* for mean weight \pm SE. (g), the allometric scaling coefficient (*b*) obtained from least-squares linear regression of log/log transformed data, and number of individuals used per treatment (*n*).

		Acclimation temperature			20 °C	
Temperature treatment	Measurement temperature	10 °C	15 °C	20 °C	25 °C	25 °C
	Mean \pm SE body weight (g)	<i>B. medius</i>	2.84 \pm 0.28	2.37 \pm 0.28	2.66 \pm 0.38	2.84 \pm 0.34
	<i>B. lesleyae</i>	0.75 \pm 0.11	0.54 \pm 0.09	0.80 \pm 0.10	0.59 \pm 0.05	0.65 \pm 0.08
scaling coefficient <i>b</i>	<i>B. medius</i>	0.29	0.88	0.56	0.89	0.43
	<i>B. lesleyae</i>	0.73	0.64	0.62	0.75	0.80
<i>n</i>	<i>B. medius</i>	10	13	12	13	11
	<i>B. lesleyae</i>	8	13	13	13	12

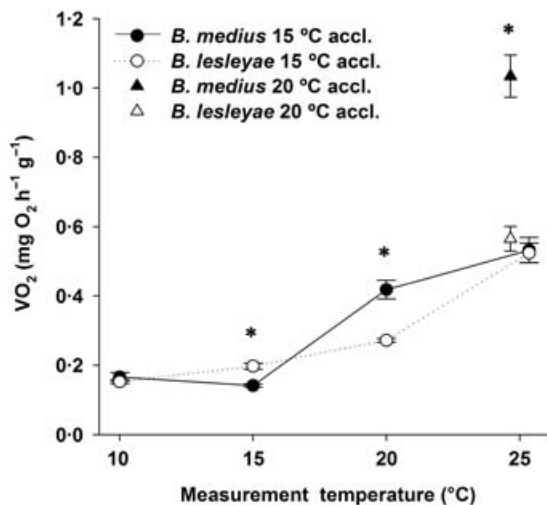


Fig. 6. Oxygen consumption (VO_2 , $mg\ O_2\ h^{-1}\ g^{-1}$) in relation to measurement and acclimation temperature (accl., °C) in *B. lesleyae* (white) and *B. medius* (black) after acclimation at 15 °C (circles) or 20 °C (triangles). Asterisk (*) indicates a significant difference between species at the relevant temperature.

experienced temperatures roughly equal to the local SST ranges which are 13–15 °C in winter (August), 15–18 °C in spring (November) and 18–23 °C in summer (February) (Leigh Marine Laboratory Climate Data Archives). Representative temperature fluctuations over a 2 day period during summer and winter in the upper monitored rockpool containing *B. medius* are shown in Fig. 5b and c.

RESPIRATORY PHYSIOLOGY

Oxygen consumption (VO_2) in relation to temperature

Species- and temperature-specific allometric scaling coefficients of VO_2 are presented in Table 1. VO_2 in both species was strongly influenced by temperature ($P < 0.001$, d.f. = 3), and VO_2 increased with rising temperature to give an overall Q_{10} (10–25 °C) of *c.* 2.2 (Fig. 6). The species did not differ in overall VO_2 across the temperature range of 10–25 °C when acclimated to 15 °C ($P = 0.35$, d.f. = 1), however, a highly significant interaction effect between species and temperature was detected ($P < 0.001$, d.f. = 3). Tukey's HSD comparisons

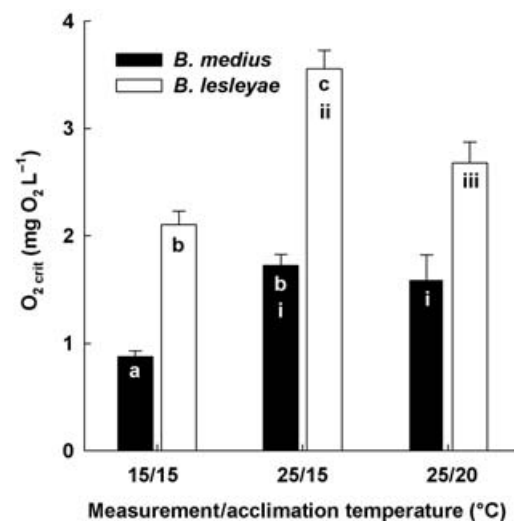


Fig. 7. Critical oxygen concentration ($O_{2\text{crit}}$; $mg\ O_2\ L^{-1}$) of *B. lesleyae* (white) and *B. medius* (black) vs. measurement temperature and acclimation temperature (°C). Points sharing the same letter or roman numeral are not significantly different.

showed that VO_2 of the two species differed significantly at 15 °C ($P < 0.001$) and 20 °C ($P < 0.001$), but not at 10 °C ($P = 1$) or 25 °C ($P = 1$, Fig. 6). Furthermore, for both species, respiration rates increased significantly only above 15 °C, with no significant difference between rates at 10 and 15 °C ($P = 0.71$; Fig. 6).

Acclimation temperature significantly affected the respiration rate at 25 °C for *B. medius* ($P < 0.001$, d.f. = 1), but not for *B. lesleyae* ($P = 0.86$, d.f. = 1, Fig. 6). The difference in species' response to acclimation temperature was indicated by a highly significant interaction effect ($P < 0.001$, d.f. = 1). When acclimated to 20 °C there was a significant difference in respiration rates between the two species at 25 °C ($P < 0.001$, d.f. = 1). Initial trials run at 30 °C showed that neither species could tolerate this temperature for long enough to run experiments even under normoxia.

Critical oxygen concentration ($O_{2\text{crit}}$)

There were significant differences in $O_{2\text{crit}}$ between the two species at both experimental temperatures ($P < 0.001$, d.f. = 1, Fig. 7). The $O_{2\text{crit}}$ of *B. medius* was $0.876 \pm 0.05\ mg\ L^{-1}$ at

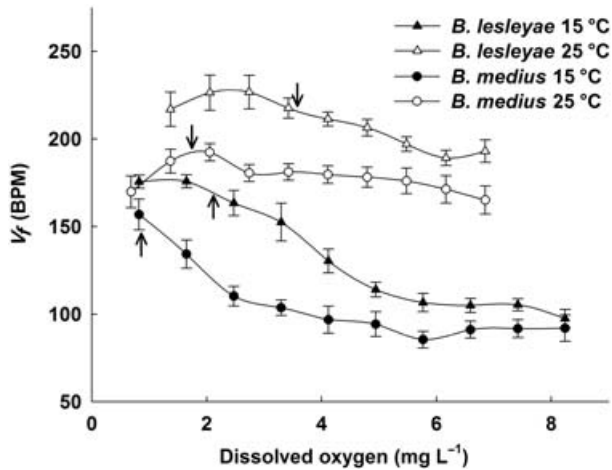


Fig. 8. Mean \pm SE opercular ventilation frequency (V_f) as beats per minute (BPM) of *B. medius* (circles) and *B. lesleyae* (triangles) in relation to dissolved oxygen concentration (mg L^{-1}) at 15 °C (black) and 25 °C (white) measurement temperatures. All fishes were acclimated at 15 °C. Arrow indicates critical oxygen concentration ($\text{O}_{2\text{crit}}$) for each species at each temperature. *B. lesleyae* 15 °C $n = 4$, 25 °C $n = 9$; *B. medius* 15 °C $n = 7$, 25 °C $n = 11$.

15 °C ($n = 12$), and $1.72 \pm 0.10 \text{ mg L}^{-1}$ at 25 °C ($n = 10$). The $\text{O}_{2\text{crit}}$ was higher in *B. lesleyae* at $2.10 \pm 0.13 \text{ mg L}^{-1}$ at 15 °C ($n = 10$), and $3.55 \pm 0.17 \text{ mg L}^{-1}$ at 25 °C ($n = 8$). The difference in $\text{O}_{2\text{crit}}$ between temperatures was also highly significant for both species ($P < 0.001$, d.f. = 1), indicating that an increase in temperature reduced the ability to oxyregulate at low O_2 tensions. A significant interaction effect ($P < 0.01$, d.f. = 1) indicated that the two species had a different magnitude of response to temperature. Tukey's comparisons indicated significant differences between all comparisons ($P < 0.01$) except for *B. lesleyae* at 15 °C vs. *B. medius* at 25 °C ($P = 0.17$, Fig. 7).

An increase in the acclimation temperature from 15 to 20 °C resulted in a significant reduction of the $\text{O}_{2\text{crit}}$ measured at 25 °C in *B. lesleyae* ($P = 0.02$, $n = 12$) but not *B. medius* ($P = 0.96$, $n = 11$) indicating that previous thermal history may be important in determining the $\text{O}_{2\text{crit}}$. *Bellapiscis medius* had a significantly lower $\text{O}_{2\text{crit}}$ than *B. lesleyae* at both acclimation temperatures (15 °C: $P < 0.001$; 20 °C: $P = 0.001$, Fig. 7).

Ventilation frequency (V_f)

At 15 °C, both species had similar V_f at normoxia of around 75–85 BPM (Fig. 8). As O_2 levels decreased, the species showed similar overall responses in V_f but differed in their timing of the response. Both species increased V_f steadily until a maximum was reached at or near the $\text{O}_{2\text{crit}}$. This V_f was maintained until the animal exhibited the 'frantic escape behaviour' and was released. At 25 °C both *B. medius* and *B. lesleyae* showed an elevated V_f under normoxia with frequencies of $c. 165 \text{ BPM}$ and 190 BPM , respectively (Fig. 8). The Q_{10} of V_f between 15 and 25 °C under normoxia was 1.8 in *B. medius* and 3.0 in *B. lesleyae*. The species showed

a different pattern of increasing V_f at 25 °C. The V_f of *B. lesleyae* continued to increase even once the $\text{O}_{2\text{crit}}$ had been surpassed to a maximum V_f of 235 BPM around 2–3 $\text{mg O}_2 \text{ L}^{-1}$, but then dropped to 222 BPM at around 1 $\text{mg O}_2 \text{ L}^{-1}$ before the experiment was terminated. *Bellapiscis medius* at 25 °C initially increased V_f only slightly, but after O_2 levels fell below $c. 2.75 \text{ mg O}_2 \text{ L}^{-1}$ there was a marked increase in V_f (although much less than the magnitude of increase of *B. lesleyae*). Unlike *B. lesleyae*, after the $\text{O}_{2\text{crit}}$ was reached the V_f of *B. medius* steadily dropped to levels nearing those at the beginning of the experiment (Fig. 8).

Discussion

This study found clear evidence for vertical habitat partitioning in the sympatric sister-species pair, with *B. medius* occupying upper and *B. lesleyae* lower rockpools, respectively. Consequently, overlap between the species only occurred at intermediate heights in the intertidal (Fig. 2). Similar pronounced habitat partitioning was also found in closely-related subtidal species of triplefin fishes in New Zealand (Wellenreuther *et al.* 2007), suggesting that divergence along the habitat axis has been an important component in the evolution of this group of fishes. The present study shows that habitat divergence is coupled with physiological differences between species, thus providing a link between ecological diversification in habitat use and physiological adaptation to habitat. These results are important as they demonstrate that not only can species-specific habitat choice reduce the overlap between heterospecific individuals, but when linked with differences in physiological tolerances, also affect the performance of individuals in their respective habitats. The results of this study therefore suggest that individuals of *Bellapiscis* species actively avoid rockpools that are unsuitable for survival, and that this behaviour is mediated by differences in physiological tolerances to determine each species' realized habitat.

Rockpool height ACD was by far the most important predictor of species occupancy in rockpools, with *B. medius* occupying rockpools higher up the shore than *B. lesleyae* (Figs 2 and 3). Individuals of *B. medius* are therefore exposed to higher temperature fluctuations, and maximum temperatures, than those experienced by *B. lesleyae* in low-shore pools. Temperature had a profound effect on the rate of oxygen consumption (VO_2), ventilation frequency (V_f) and critical oxygen levels ($\text{O}_{2\text{crit}}$) in both species (Figs 6, 7 and 8). Temperature quotient (Q_{10}) values were similar between species and were $c. 2.2$ ($Q_{10} 10\text{--}25 \text{ °C}$), which is fairly typical for intertidal fishes and teleosts in general (Bridges 1988; Bridges 1993; Clarke & Johnston 1999). Attempts to measure VO_2 and $\text{O}_{2\text{crit}}$ at 30 °C indicated that 30 °C appears to be beyond both species' upper thermal tolerance limits. This result indicates that *B. medius* individuals found in the highest rockpools during this study may be living very near to their upper thermal limits in the wild. Indeed, high mortality of *B. medius* has been observed in upper-shore rockpools near the Matheson's Bay study site on a calm summer's day at low tide

(R. Taylor pers. commun.), indicating that heat or hypoxic stress may be a strong selective factor. The lack of increase in VO_2 measured at 25 °C in *B. lesleyae* with an increase in acclimation temperature from 15 to 20 °C (Fig. 8), indicates that VO_2 was also at or very near its maximum at this temperature, and that VO_2 is likely to be compromised at higher temperatures in this species. However, experiments attempted at 30 °C in this study followed acclimation at 15 °C, and it is likely, given the ability of both species to alter VO_2 and/or $O_{2\text{crit}}$ in response to acclimation temperature, that seasonal changes in temperature tolerance of VO_2 and $O_{2\text{crit}}$ may occur.

In aquatic species, increased temperature causes an increase in metabolism and hence O_2 demand, but a decrease in the O_2 availability due to a decrease in O_2 solubility with temperature. Thus fish in higher rockpools are likely to experience reduced levels of O_2 availability concurrently with higher temperatures. Results of this study indicate that differences in hypoxia tolerance may be important in partitioning these species. *Bellapiscis medius* had a significantly lower $O_{2\text{crit}}$ at both 15 and 25 °C than *B. lesleyae*, thus indicating a greater hypoxia tolerance at both temperatures (Fig. 7). Behavioural mechanisms may also play a part in hypoxia avoidance by *B. medius*, as individuals of *B. medius* occurred in rockpools with higher surface area than *B. lesleyae* on the upper-shore (> 1.17 m ACD; Fig. 3). Rockpools with higher surface areas have larger areas for diffusion of O_2 , thereby potentially reducing the likelihood and extent of hypoxia or hyperoxia. Aggregation size in *B. medius* was strongly correlated with pool surface area and pool volume, but this was not observed in *B. lesleyae*. Thus, our results suggest that occupancy of high-shore rockpools by *B. medius* may be limited by surface area to volume constraints affecting levels of dissolved gases, but this is not the case for the low-shore rockpools inhabited by *B. lesleyae*. Adult *B. medius* occurred in significantly smaller aggregations than *B. lesleyae* (Fig. 4), which is to be expected if O_2 availability is indeed limiting. Adult *B. medius* weighed on average around four times as much as *B. lesleyae*, and therefore despite having a similar VO_2 per gram, the absolute VO_2 per individual *B. medius* is around four times that of *B. lesleyae*. All other factors being equal, if O_2 were limiting, this would therefore limit the number of individuals of *B. medius* to 25% of the density of *B. lesleyae* that could occupy a rockpool of any given volume and available O_2 .

Although both species had a similar overall response of increasing V_f in response to hypoxia and temperature increase, the timing and magnitude of these responses differed between species. *B. lesleyae* appeared to be more sensitive to both hypoxia and temperature increase, displaying a greater increase of V_f with temperature as well as increasing V_f sooner in response to falling O_2 concentrations than *B. medius* (Fig. 8). This indicates that *B. medius* is more tolerant of hypoxia and temperature change than *B. lesleyae*, and these results are supported by the respective values of Q_{10} for VO_2 and the values of $O_{2\text{crit}}$. These findings therefore suggest that divergence in physiological performance has been associated with divergence in habitat choice in the *Bellapiscis* species.

Similar relationships between thermal physiology and zonation patterns have been shown previously in closely-related sympatric fishes (Matthews & Styron 1981; Brown 1989; Hölker 2006; Ohlberger *et al.* 2008). Hölker (2006) examined bream (*Abramis brama*) and roach (*Rutilus rutilus*) (Family Cyprinidae), and found small but significant interspecific differences in VO_2 which appear to reflect the relative thermal properties of their microhabitat preferences. Likewise, Brown (1989) found differences in VO_2 and temperature preferences between three sympatric species of sculpin (*Cottus*) which reflected habitat partitioning in these species, although they did not explain the absence of one species from areas occupied by the other two, suggesting competition or other biotic factors were also important. Matthews & Styron (1981) also found significant differences in physiological tolerance to acute changes in O_2 saturation, pH and temperature which matched the longitudinal zonation of sympatric species of river dwelling darter (*Etheostoma*). Ohlberger *et al.* (2008) have recently shown that differences in thermal physiology likely underpin habitat partitioning between a sympatric sister-species pair of lake dwelling coregonid fishes along a vertical temperature gradient. The results of the current study add to this growing body of literature, and indicate that physiological differences may underpin habitat divergence in sympatric sister-species of marine triplefin fishes. New Zealand triplefin fishes show pronounced and consistent habitat partitioning (Wellenreuther *et al.* 2008) which is particularly strong between pairs of sister-species (Wellenreuther *et al.* 2007). The integrative approach in the present study allowed us to show that divergence in habitat use in this sympatric sister-species pair is directly and strongly linked to physiological performance in their respective habitats. Integrative studies of physiology and ecology of closely related sympatric species like that presented here can thus offer insight into the selective pressures that may have influenced the evolutionary divergence of sister-species.

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