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RESEARCH ARTICLE

Will jumping snails prevail? Influence of near-future CO_2, temperature and hypoxia on respiratory performance in the tropical conch *Gibberulus gibberulus gibbosus*

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ABSTRACT

Tropical coral reef organisms are predicted to be especially sensitive to ocean warming because many already live close to their upper thermal limit, and the expected rise in ocean CO_2 is proposed to further reduce thermal tolerance. Little, however, is known about the thermal sensitivity of a diverse and abundant group of reef animals, the gastropods. The humpbacked conch (*Gibberulus gibberulus gibbosus*), inhabiting subtidal zones of the Great Barrier Reef, was chosen as a model because vigorous jumping, causing increased oxygen uptake ($\dot{M}_{O_2}$), can be induced by exposure to odour from a predatory cone snail (*Conus marmoreus*). We investigated the effect of present-day ambient (417–454 µatm) and projected-future (955–987 µatm) P_{CO_2} on resting ($\dot{M}_{O_2,rest}$) and maximum ($\dot{M}_{O_2,max}$) $\dot{M}_{O_2}$, as well as $\dot{M}_{O_2}$ during hypoxia and critical oxygen tension ($P_{O_2,crit}$), in snails kept at present-day ambient (28°C) or projected-future temperature (33°C). $\dot{M}_{O_2,rest}$ and $\dot{M}_{O_2,max}$ were measured both at the acclimation temperature and during an acute 5°C increase. Jumping caused a 4- to 6-fold increase in $\dot{M}_{O_2}$, and $\dot{M}_{O_2,max}$ increased with temperature so that absolute aerobic scope was maintained even at 38°C, although factorial scope was reduced. The humpbacked conch has a high hypoxia tolerance with a $P_{O_2,crit}$ of 2.5 kPa at 28°C and 3.5 kPa at 33°C. There was no effect of elevated CO_2 on respiratory performance at any temperature. Long-term temperature records and our field measurements suggest that habitat temperature rarely exceeds 32.6°C during the summer, indicating that these snails have aerobic capacity in excess of current and future needs.

KEY WORDS: Aerobic scope, Global warming, Climate change, Ocean acidification, Gastropod, Mollusc

INTRODUCTION

Ocean temperature and carbon dioxide (CO_2) levels are rising and this is projected to continue throughout this century (IPCC, 2013). A huge research effort is currently devoted to studying the effects of these changes on aquatic life, with the main focus on organisms that may be at particular risk, such as calcifying marine invertebrates and coral reef fish (Hoegh-Guldberg et al., 2007; Munday et al., 2012; Pörtner et al., 2014). Rising ocean temperatures have been predicted to negatively influence fitness of aquatic ectotherms because the scope for aerobic metabolism is hypothesized to have an optimal temperature (Fry, 1947; Fry and Hart, 1948), below and above which the capacity for activity, growth and reproduction will be reduced (Wang and Overgaard, 2007). The aerobic scope indicates how much oxygen is available for processes beyond basic maintenance. It can be expressed either as absolute aerobic scope (AAS), which is the difference in oxygen uptake ($\dot{M}_{O_2}$) between the maximum ($\dot{M}_{O_2,max}$) and minimum ($\dot{M}_{O_2,min}$) (AAS=$\dot{M}_{O_2,max}$− $\dot{M}_{O_2,min}$), or as factorial aerobic scope (FAS), which is the proportional difference (FAS=$\dot{M}_{O_2,max}$/$\dot{M}_{O_2,min}$) (e.g. Clark et al., 2013). Hypoxia, high CO_2, and thereby low pH, may act to narrow the temperature range where aerobic scope is maintained (e.g. Pörtner et al., 2005; Rosa and Seibel, 2008; Pörtner and Farrell, 2008; Dissanayake and Ishimatsu, 2011). Additionally, elevated temperature may reduce not only aerobic scope but also hypoxia tolerance (measured as the critical oxygen tension, $P_{O_2,crit}$) (e.g. Fry, 1947; Fry and Hart, 1948), as shown for some coral reef fish (Nilsson et al., 2010) and cuttlefish (Rosa et al., 2013). In the latter case, there was even a synergistic effect of CO_2, increasing $P_{O_2,crit}$ further at elevated temperature. Consequently, the importance of testing the combined effects of warming and ocean acidification is increasingly acknowledged in efforts to predict the impact of climate change on marine ecosystems (Harvey et al., 2013; Gaylord et al., 2015).

Organisms in tropical areas, such as the Great Barrier Reef, are predicted to be particular sensitive to ocean warming, because they may already be living at the edge of their metabolic capacity (e.g. Johansen and Jones, 2011; Nguyen et al., 2011; Rummer et al., 2014). For several coral reef fish it has been confirmed that aerobic scope is negatively impacted by projected future temperatures (e.g. Nilsson et al., 2009; Munday et al., 2012; Rummer et al., 2014). It has also been shown in some of these species that elevated CO_2 can exacerbate this effect (Munday et al., 2009), although in other reef fishes elevated CO_2 may have a positive effect on aerobic scope (Couturier et al., 2013; Rummer et al., 2013). A huge effort has also gone into investigating the impact of ocean acidification on calcifying animals like corals, molluscs and crustaceans, as they are likely to be directly affected by the declining saturation state of carbonate ions that accompanies ocean acidification (Hofmann et al., 2010; Parker et al., 2013). However, to our knowledge there have been no studies on potential interacting effects of increased temperature and CO_2 on the aerobic scope of tropical coral reef invertebrates.

Studies of thermal tolerance of intertidal gastropods, mainly temperate water species, have a long history. This group generally has high critical temperatures that tend to correlate well with their vertical distribution in the intertidal environment (e.g. Southward, 1958; Simpson, 1976; Underwood, 1979; Somero, 2002; Salas et al., 2014). In some intertidal snails, particularly from the clade Littorinimorpha, the tolerance extends to metabolism, as the increase in resting $\dot{M}_{O_2}$ ($\dot{M}_{O_2,rest}$) with temperature only breaks down at very high temperatures (e.g. 35°C, Newcombe et al., 1936;

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List of symbols and abbreviations

AAS	absolute aerobic scope
β_{w,O_2}	capacitance coefficient of oxygen in water
EPOC	excess post-exercise oxygen consumption
FAS	factorial aerobic scope
IMOS	Integrated Marine Observing System
M_b	wet tissue body mass
M_{O_2}	total mass-specific oxygen uptake or consumption
$\dot{M}_{O_2}$	mass-specific rate of oxygen uptake or consumption
$\dot{M}_{O_2,max}$	mass-specific maximum rate of oxygen uptake or consumption
$\dot{M}_{O_2,min}$	mass-specific minimum or standard rate of oxygen uptake or consumption
$\dot{M}_{O_2,rest}$	mass-specific resting rate of oxygen uptake or consumption
P_{CO_2}	carbon dioxide partial pressure
P_{O_2}	oxygen partial pressure
$P_{O_2,crit}$	critical oxygen tension (partial pressure)
Q_{10}	temperature coefficient

37°C, Lewis, 1971; 55°C, Marshall et al., 2011). This high temperature tolerance of intertidal gastropods is probably related to their habitat, where both very high temperatures and hypoxia due to air exposure may occur on a daily basis (e.g. Lewis, 1963; Truchot and Duhamel-Jouve, 1980; Helmuth et al., 2006), although overall temperature ranges are obviously dependent on the latitudinal location. There is generally less known about the temperature tolerance of tropical marine gastropods, especially in species that are less likely to experience air exposure because they inhabit the subtidal rather than the intertidal environment.

The humpbacked conch *Gibberulus gibberulus gibbosus* (Röding 1798), which is abundant in subtidal zones of the Great Barrier Reef, is an ideal gastropod for studying the effect of increased ocean temperature and CO_2 partial pressure (P_{CO_2}) on aerobic performance, because it can be exercised. Like some of its relatives in the family Strombidae (Parker, 1922), this snail is able to jump or leap, using its foot as a 'muscular hydrostat' (Kier, 2012). Jumping causes a substantial increase in $\dot{M}_{O_2}$ (Watson et al., 2014), and $\dot{M}_{O_2}$ during this state of vigorous exercise can be assumed to be close to $\dot{M}_{O_2,max}$, as it is with the 'chase protocol' used to estimate $\dot{M}_{O_2,max}$ in fish (e.g. Reidy et al., 1995; Clark et al., 2013). The humpbacked conch uses its ability to jump to escape from predators, such as the marbled cone snail *Conus marmoreus* Linnaeus 1758 (Watson et al., 2014). The response is mediated by olfaction and jumping is therefore readily triggered in experiments by exposing snails to cone snail-scented water (Kohn, 1961; Kohn and Waters, 1966; Field, 1977; Watson et al., 2014). By measuring $\dot{M}_{O_2}$ during both resting and jumping, aerobic scope can be estimated under a variety of environmental conditions.

The aims of this study were to: (1) investigate whether the humpbacked conch maintains aerobic scope at high temperatures, as could be expected if it possesses the heat tolerance shown by other gastropods, and (2) investigate whether aerobic scope is reduced at high temperature when combined with elevated CO_2, even if it is maintained under present-day ambient CO_2 conditions. Additionally, we tested: (3) whether the humpbacked conch has a high hypoxia tolerance, by possessing a low $P_{O_2,crit}$, as could be expected from its habitat and (4) whether $P_{O_2,crit}$ is increased, and thereby hypoxia tolerance reduced, after 1–3 weeks exposure to either elevated temperature or elevated CO_2, or both.

We characterized the respiratory capacities of the humpbacked conch at elevated temperature combined with elevated CO_2. Firstly, aerobic scope was measured in snails that had been held for more than 1 week at two temperatures, 28 and 33°C (referred to as 28 and 33°C-acclimated snails, respectively), which represent the present-day ambient and a projected future summer temperature for the Great Barrier Reef (Hennessy et al., 2007). Additionally, snails were exposed to an acute 5°C increase (i.e. to 33°C for 28°C-acclimated snails and to 38°C for 33°C-acclimated snails, referred to as acute-33°C snails and acute-38°C snails, respectively). These acute exposures were included because we expected that these snails, even if they live in the subtidal zone, may experience rapid temperature rises if the shallow water is excessively heated by the sun during daytime. Measurements at all temperatures were done on snails acclimated to either ambient or elevated P_{CO_2} (referred to as ambient CO_2 and elevated CO_2, respectively). The elevated-CO_2 treatment (950–990 µatm=0.096–0.100 kPa P_{CO_2}) was consistent with projections for CO_2 levels in the surface ocean by year 2100 based on representative concentration pathway (RCP)8.5 (Meinshausen et al., 2011; Doney et al., 2012). Secondly, as hypoxia may be experienced by snails when they burrow in the sand (or if they on rare occasions are emersed), and as hypoxia is projected to become more common in a warmer future (Diaz, 2001; Matear and Hirst, 2003; Diaz and Rosenberg, 2008), $P_{O_2,crit}$ was determined in order to evaluate hypoxia tolerance and ability to regulate oxygen uptake independently of oxygen partial pressue (P_{O_2}). This was done at both treatment temperatures and CO_2 levels, but not during an acute exposure to increased temperature. Lastly, temperatures were monitored in both the sand and the water of the snails' habitat in the peak of the summer, and available data on daily maximum temperature in the Lizard Island lagoon over the past 15 years were consulted [Australian Institute of Marine Science (AIMS), 2015; Integrated Marine Observing System (IMOS), 2015a], to determine the upper range of present-day ambient temperatures in their natural environment.

RESULTS

Respiratory performance

Oxygen uptake showed a consistent response in all treatment groups (Fig. 1). $\dot{M}_{O_2}$ rose immediately after jumping was induced by injecting cone snail odour into the respirometers. This was followed by a gradual decline in $\dot{M}_{O_2}$ after the jumping ceased and odour was flushed out. Finally, $\dot{M}_{O_2}$ stabilized at a low level after 5–12 h, indicating that the snail had recovered from the jumping activity and entered a resting state. The effect of time on $\dot{M}_{O_2}$ was highly significant at 28°C (Fig. 1A; two-way ANOVA with repeated measures, $F_{20,380}$=81.64, P<0.0001), acute-33°C (Fig. 1B; $F_{20,400}$=71.23, P<0.0001), 33°C (Fig. 1C; $F_{20,340}$=57.79, P<0.0001) and acute-38°C (Fig. 1D; $F_{20,320}$=124.9, P<0.0001). The response was not affected by CO_2 at 28°C ($F_{1,19}$=0.5976, P=0.4490), acute-33°C ($F_{1,20}$=1.227, P=0.2812), 33°C ($F_{1,17}$=0.2768, P=0.6056) or acute-38°C ($F_{1,16}$=0.0091, P=0.9254). There were no differences between treatment groups in jumping rate itself (Table 1; one-way ANOVA, $F_{7,89}$=1.175, P=0.3250) or in the oxygen consumed per jump, the 'jump cost' (Table 1; $F_{7,67}$=1.046, P=0.4081). Likewise, the excess post-exercise oxygen consumption (EPOC or 'oxygen debt') was not significantly different between treatment groups (Table 1; $F_{7,60}$=1.432, P=0.2095). There was a significant linear relationship between total number of jumps and total amount of oxygen consumed during jumping (Fig. 2; linear regression, $F_{1,73}$=98.96, P<0.0001, R^2=0.5755).

Overall, $\dot{M}_{O_2,rest}$ (Fig. 3A) increased with temperature, and there was a significant difference between treatment groups (one-way ANOVA, $F_{7,72}$=23.52, P<0.0001). Specifically, 28°C-acclimated snails increased $\dot{M}_{O_2,rest}$ with a temperature coefficient (Q_{10}) of 2.0

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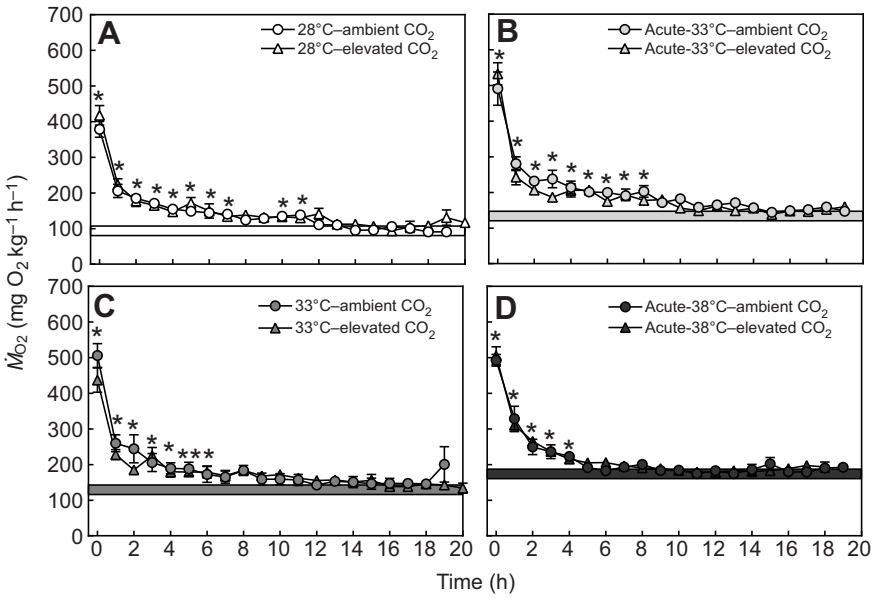

Fig. 1. Oxygen uptake ($\dot{M}_{O_2}$) during and after jumping. Data (means±s.e.m.) are for (A) 28°C snails in ambient CO_2 (N=11) or elevated CO_2 (N=8); (B) acute-33°C snails in ambient CO_2 (N=11) or elevated CO_2 (N=10); (C) 33°C snails in ambient CO_2 (N=8) or elevated CO_2 (N=10); and (D) acute-38°C snails in ambient CO_2 (N=6) or elevated CO_2 (N=12). Snails were measured at either their treatment temperature (28 or 33°C) or acutely 5°C above (for 28°C snails, acute-33°C; and for 33°C snails, acute-38°C). Snails jumped only at 0 h. Horizontal bars indicate resting oxygen uptake ($\dot{M}_{O_2,rest}$; means±s.e.m.). Asterisks indicate a significant elevation above $\dot{M}_{O_2,rest}$ for both ambient and elevated CO_2. There was no effect of CO_2 at any temperature (see Results for details).

when acutely exposed to 33°C (Šídák's multiple comparisons test, P<0.0001 for both ambient and elevated CO_2), and 33°C-acclimated snails increased $\dot{M}_{O_2,rest}$ with a Q_{10} of 1.8 when acutely exposed to 38°C (P=0.0035 for ambient CO_2 and P=0.0002 for elevated CO_2). The $\dot{M}_{O_2,rest}$ of 33°C-acclimated snails was still significantly higher than that of 28°C-acclimated snails (Q_{10}=1.9) in both ambient CO_2 (P<0.0001) and elevated CO_2 (P=0.0003). Accordingly, there was no difference in $\dot{M}_{O_2,rest}$ between 28 and 33°C-acclimated snails when both were measured at 33°C (P>0.9999 for both ambient and elevated CO_2). Lastly, there was no significant effect of CO_2 at any temperature on $\dot{M}_{O_2,rest}$ (P>0.9999 at all four measurement temperatures).

$\dot{M}_{O_2,max}$ (Fig. 3B) was affected by temperature treatment (one-way ANOVA, $F_{7,69}$=5.474, P<0.0001). Specifically, $\dot{M}_{O_2,max}$ of 28°C-acclimated snails was higher when tested acutely at 33°C (acute-33°C) than at 28°C in snails from both ambient CO_2 (Šídák's multiple comparisons test, P=0.0006) and elevated CO_2 (P=0.0159). $\dot{M}_{O_2,max}$ of 33°C-acclimated snails was maintained when tested acutely at 38°C in both ambient CO_2 (P>0.9999) and elevated CO_2 (P=0.4319). There was also a tendency for $\dot{M}_{O_2,max}$ of 33°C-acclimated snails to be higher than $\dot{M}_{O_2,max}$ of 28°C-acclimated snails, but the effect was only significant in ambient CO_2 (P=0.004 for ambient CO_2 and P>0.9999 for elevated CO_2). There was no significant difference between snails acclimated to 28 and 33°C when both were measured at 33°C, in either ambient CO_2 (P>0.9999) or elevated CO_2 (P=0.1176). Furthermore, there was no effect of CO_2 at 28°C (P=0.9993), acute-33°C (P>0.9999), 33°C (P=0.2198) or acute-38°C (P>0.9999).

Following a similar pattern, there was a small but significant effect of treatment on AAS (Fig. 3C; one-way ANOVA, $F_{7,67}$=3.568, P=0.0025). AAS of 28°C-acclimated snails in ambient CO_2 was significantly higher when tested acutely at 33°C (Šídák's multiple comparisons test, P=0.0209), but the effect was not significant for snails tested in elevated CO_2 (P=0.2362). AAS of 33°C-acclimated snails was not reduced at 38°C in either ambient CO_2 (P=0.6231) or elevated CO_2 (P=0.9903). AAS also tended to be higher in 33°C snails compared with 28°C snails at ambient CO_2 (P=0.0582) but not in elevated CO_2 (P>0.9999). In elevated-CO_2 snails there was a tendency for AAS of 33°C snails to be lower than that of 28°C snails when measured at 33°C (P=0.0727), but this was not the case in ambient CO_2 (P>0.9999). Overall, there were no significant effects of CO_2 (P>0.9999 for 28°C, acute-33°C and acute-38°C) on AAS. The tendency for AAS to be lower in 33°C-acclimated snails in elevated CO_2 compared with ambient CO_2 was not significant (P=0.0895).

There was an overall significant effect of temperature treatment on FAS (Fig. 3D; one-way ANOVA, $F_{7,67}$=10.65, P<0.0001). FAS was not reduced in 28°C-acclimated snails when measured at acute-33°C in either ambient CO_2 (P>0.9999) or elevated CO_2 (P=0.9449). It was significantly lower at acute-38°C compared with 33°C in ambient-CO_2 snails (P=0.0013), but not in elevated-CO_2 snails (P=0.5182). In contrast, FAS was lower in 33°C-acclimated snails than in 28°C-acclimated snails in elevated CO_2 (P=0.009), but not in ambient CO_2 (P>0.9999). Furthermore, there was no effect of acclimation, as FAS of 28°C-acclimated snails measured acutely at 33°C was not different

Table 1. Jumping rate, cost per jump and excess post-exercise oxygen consumption (EPOC)

Acclimation temperature	Measurement temperature	P_{CO_2} group	Jumping rate (min^{-1})	Cost per jump (mg O_2 kg^{-1} $jump^{-1}$)	EPOC (mg O_2 kg^{-1})
28°C	28°C	Ambient	19±3 (12)	0.31±0.05 (10)	435±61 (9)
		Elevated	16±2 (12)	0.38±0.04 (10)	522±71 (9)
	Acute-33°C	Ambient	19±2 (13)	0.41±0.05 (9)	609±80 (8)
		Elevated	23±3 (12)	0.36±0.06 (11)	616±111 (10)
33°C	33°C	Ambient	22±2 (12)	0.34±0.05 (8)	516±99 (8)
		Elevated	20±2 (12)	0.27±0.02 (9)	389±105 (6)
	Acute-38°C	Ambient	19±2 (12)	0.33±0.05 (6)	313±92 (7)
		Elevated	21±1 (12)	0.29±0.02 (12)	446±57 (11)

Data are means±s.e.m. Sample sizes are given in parentheses. There were no significant differences between groups (see Results for details).

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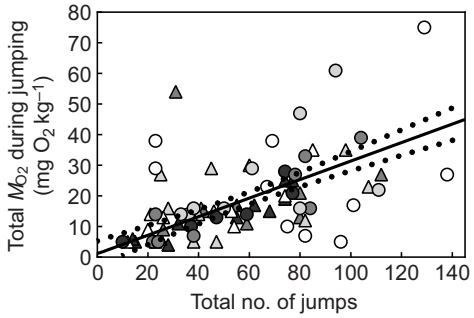

Fig. 2. Total M_{O_2} during jumping as a function of total number of jumps. Points represent individual snails exposed to ambient CO_2 (circles) or elevated CO_2 (triangles) at 28°C (white), acute-33°C (light grey), 33°C (grey) or acute-38°C (dark grey). Dotted lines represent the 95% confidence interval. As there was no significant difference between the eight measurement groups (see Results for details), the solid line represents a linear regression fitted to the pooled dataset (75 points, M_{O_2}=0.30×jumps+1.19).

from FAS of 33°C snails in either ambient CO_2 (P>0.9999) or elevated CO_2 (P=0.2118). There was no significant effect of elevated CO_2 at 28°C (P>0.9999), acute-33°C (P=0.9987), 33°C (P=0.1128) or acute-38°C (P>0.9999).

$P_{O_2,crit}$

Upon exposure to gradual hypoxia (Fig. 4A), $\dot{M}_{O_2}$ decreased slowly from higher P_{O_2} levels down to 5.3 kPa, where it started to fall more rapidly until just below 2.7 kPa, at which point $\dot{M}_{O_2}$ became directly and linearly dependent on water P_{O_2}. $P_{O_2,crit}$ was only slightly, but nonetheless significantly, elevated with temperature (Fig. 4B; two-way ANOVA, $F_{1,30}$=37.72, P<0.0001) in both ambient CO_2 (P=0.0009) and elevated CO_2 (P<0.0001), but there was no effect of CO_2 ($F_{1,30}$=0.003698, P=0.9519) at either 28°C (P=0.9323) or 33°C (P=0.9571).

Temperature in the habitat

There was no or very minor cloud cover during the days of temperature measurements in the field (12–16 December 2013). The sampling dates were also close to the Austral midsummer (21 December) and within the warmest and calmest period of the year before the rainy season, which generally begins in January (AIMS, 2015; IMOS, 2015b). Light intensity measurements from the sensors (Fig. 5A) confirmed that two sensors remained buried in the sand for the 4 days of measurement (light intensity was zero), while one sensor was in the water with light intensity showing diurnal variation. Only one of the sites were periodically exposed to air (site A; see supplementary material Fig. S1), when the tide was low (Fig. 5B), and this occurred during the night when the temperature was lower. Generally, the temperature in both sand and water, independent of water depth, approached the air temperature during the night (Fig. 5C), while the temperature varied more during the day. The highest temperature was reached in the water at site B_W (32.5°C), while the maximum temperature was lower in both sand sites (~29.5–30.0°C). The measurements also confirmed that the snails can experience a daily change in temperature of up to 5°C (Fig. 5C).

In the past 5 years, during which water temperature has been monitored at 0.6 m below the surface in the Lizard Island lagoon, the daily maximum temperature has only exceeded 31°C on 0.7% of the days. The maximum water temperature ever recorded in the past 5 years is 32.6°C, and the maximum daily change recorded during this period is 3.3°C (supplementary material Fig. S2A; IMOS, 2015a). During the period from 1995 to 2012, daily maximum temperature was also monitored at 2.1 m depth on a reef flat (i.e. shallow water with a temperature profile more closely resembling the humpbacked conch habitat) in the Lizard Island lagoon, and during this 17 year period the temperature only exceeded 32°C on 0.5% of the days. The maximum temperature ever recorded in this period was 33.3°C while the maximum daily change recorded was 5°C (supplementary material Fig. S2B; AIMS, 2015).

DISCUSSION

General aspects of aerobic performance of the humpbacked conch

The immediate response to predator odour was a 4- to 5-fold increase in $\dot{M}_{O_2}$ – in some individuals, it was closer to 6-fold. Compared specifically with other gastropods, this factorial scope is

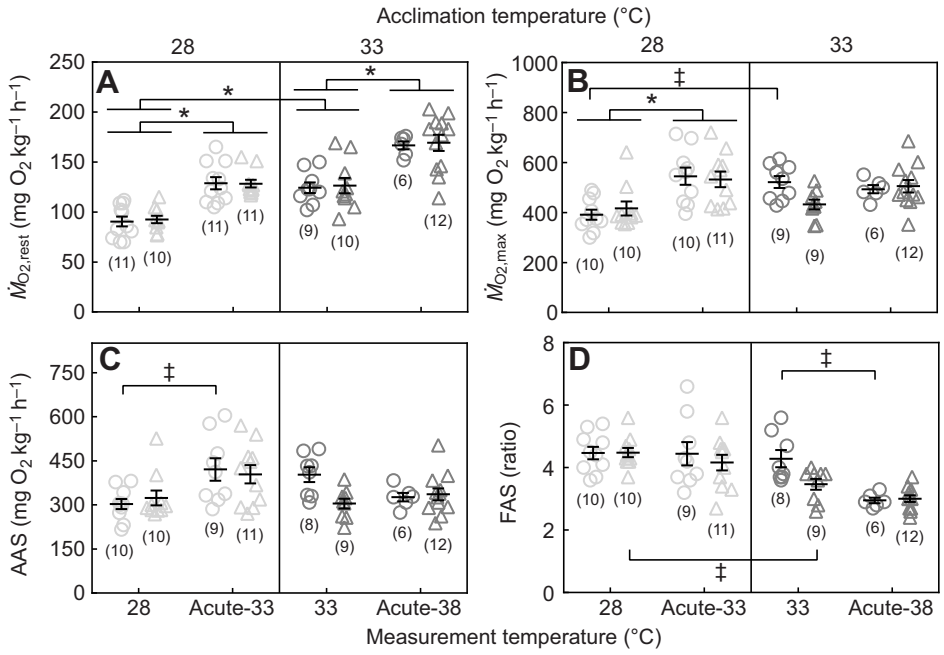

Fig. 3. Effect of elevated CO_2 and temperature on aerobic performance. Resting oxygen uptake ($\dot{M}_{O_2,rest}$; A), maximum oxygen uptake ($\dot{M}_{O_2,max}$; B), absolute aerobic scope (AAS; C) and factorial aerobic scope (FAS; D) of snails acclimated to either 28 or 33° C in combination with ambient CO_2 (circles) or elevated CO_2 (triangles). Snails were measured at either their treatment temperature (28 or 33°C) or acutely 5°C above (28°C snails at acute-33°C and 33°C snails at acute-38°C). Data points represent individual snails and solid lines represent the mean±s.e.m. Sample sizes are given in parentheses. Asterisks indicate a significant difference between two temperatures at both CO_2 levels. Double daggers indicate a significant difference between two temperatures only at a particular CO_2 level. There was no effect of CO_2 on any parameter at any temperature (see Results for details).

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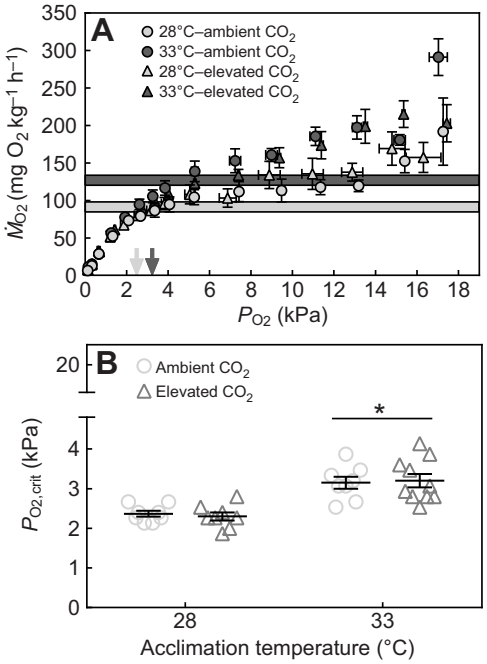

Fig. 4. Effect of elevated CO_2 and temperature on the response to gradual hypoxia. $\dot{M}_{O_2}$ as a function of water oxygen partial pressure (P_{O_2}; A) and calculated critical oxygen tension ($P_{O_2,crit}$; B) in snails acclimated to 28 or 33°C at ambient or elevated CO_2. In A, mean $\dot{M}_{O_2,rest}$ is indicated with horizontal bars and mean $P_{O_2,crit}$ is indicated by arrows (light grey for 28°C-acclimated snails and dark grey for 33°C-acclimated snails). N=8, except for the 33°C–elevated-CO_2 snails, where N=10. Both $\dot{M}_{O_2}$ and P_{O_2} data are means±s.e.m. In B, data points represent $P_{O_2,crit}$ of individual snails and solid lines represent means±s.e.m. An asterisk indicates a significant effect of temperature at both CO_2 levels. There was no effect of CO_2 at either temperature (see Results for details).

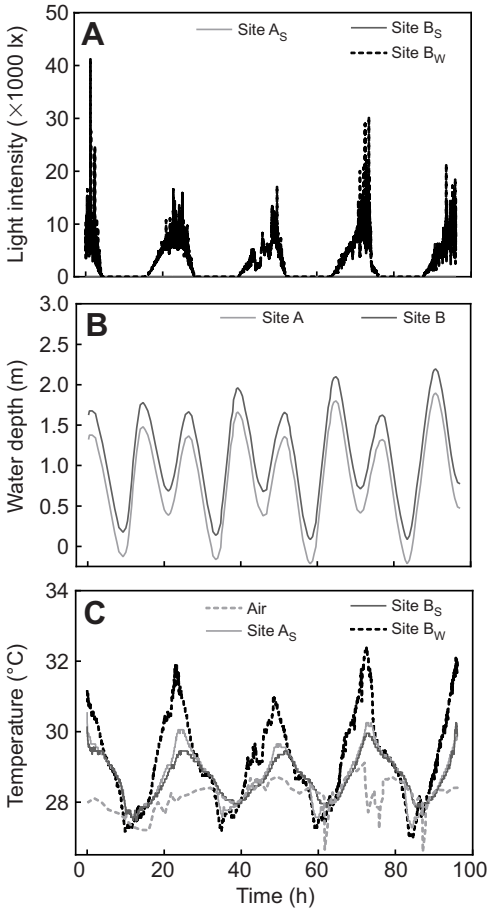

Fig. 5. Temperature, light and water depth at the snail collection site. Light intensity (A), water depth (B) and temperature (C) as a function of time, at different sites either 5 cm above the sand bottom (B_W) or buried 5 cm into the sand (sites A_S and B_S). See supplementary material Fig. S1. Site A was located closer to the shore than site B, with an approximate 0.3 m difference in water depth. Air temperature in C was obtained from the Integrated Marine Observing System (IMOS) at Lizard Island (Australian Institute of Marine Science) (see IMOS, 2015b).

among the highest recorded, although it may not be unique as studies on tropical molluscs are scarce (Seebacher et al., 2015). For example, the plough snail (*Bullia digitalis*) has a FAS of around 4 (15°C-acclimated snails measured at 25°C; Brown and da Silva, 1983), while the common periwinkle (*Littorina littorea*) has a FAS of only around 2 (measured at 30°C; Newell, 1973). Indeed, the FAS of the humpbacked conch is comparable to that of many fishes (e.g. Killen et al., 2007; Nilsson et al., 2009; Lefevre et al., 2014a), which have a much more active lifestyle. The smaller tropical coral reef fish, however, tend to have lower scopes ranging from 1.5 to 3.5 in some damselfishes and 3 to 4.5 in some cardinalfishes (Nilsson et al., 2009; Gardiner et al., 2010; Donelson et al., 2012). The $\dot{M}_{O_2,max}$ of 375–600 mg O_2 kg^{-1} h^{-1} and AAS of 300–450 mg O_2 kg^{-1} h^{-1} reported here are also amongst the highest recorded in gastropods (Brown and da Silva, 1983; Carefoot, 1989). These values are nonetheless lower than in some of the coral reef fish, where AAS ranges from 300 to 1250 mg O_2 kg^{-1} h^{-1} (Nilsson et al., 2009; Gardiner et al., 2010; Rummer et al., 2014). This probably reflects the lifestyle of small coral reef fishes, where high competition for food and other resources in a complex habitat, and a high predation pressure make a high aerobic scope beneficial. The humpbacked conch, in contrast, while capable of performing intense activity in particular situations, namely during an escape response, may be unable to reach the very high rates of oxygen uptake displayed by some fishes in the same habitat, because of its relatively primitive open circulatory system. Still, any general conclusions in this respect may be premature as studies of active metabolism in tropical gastropods, and even tropical molluscs in general, are much neglected areas of research.

The fact that $\dot{M}_{O_2}$ increased immediately when predator-scented water was injected into the respirometer indicates that jumping is at least partially fuelled by aerobic metabolism. It is possible that the predator odour induced a stress response causing an elevation in $\dot{M}_{O_2}$, but the linear relationship between total M_{O_2} during jumping and the total number of jumps indicates that the majority of the variation in $\dot{M}_{O_2}$ after exposure to predator odour is explained directly by jumping activity. Furthermore, if stress itself was the major component of the increase in $\dot{M}_{O_2}$ during jumping, we would have expected a larger positive intercept when total M_{O_2} was extrapolated back to zero jumps. However, after a few minutes and a varying number of jumps, the snails stopped jumping, which could indicate fatigue, possibly related to a build up of end products from anaerobic metabolism. This conclusion is supported by the presence of EPOC up to 12 h after a jumping bout, although measurements of anaerobic end products would be necessary to confirm this. The relative contribution of anaerobic and aerobic metabolism in relation to locomotion in gastropods has been the focus of several studies, and appears to vary widely between different species, as well as with the nature and intensity of the activity investigated (Baldwin and Lee, 1979; Baldwin and England, 1982; Donovan et al., 1999). Anaerobic metabolism in gastropods is in itself very

diverse, with several pathways for anaerobic metabolism (e.g. Santini et al., 2001; see review by Livingstone, 1983), resulting in a variety of possible end products in addition to lactate (Baldwin and Opie, 1978; Gäde, 1988; Behrens et al., 2002). Such studies, however interesting, were beyond the scope of the present work.

$P_{O_2,crit}$

Animals are often classified as oxy-conformers if their $\dot{M}_{O_2}$ is directly dependent on P_{O_2} over the entire P_{O_2} range, or as oxy-regulators if they are able to maintain $\dot{M}_{O_2,rest}$ independent of P_{O_2} down to a critical O_2 level, the $P_{O_2,crit}$, whereupon they become oxy-conformers (e.g. Tang, 1933; Fry and Hart, 1948; Mueller and Seymour, 2011). The degree of oxy-conformity can vary, in particular with temperature (Berg et al., 1962; Newell et al., 1978; Alexander and McMahon, 2004). $\dot{M}_{O_2}$ in the humpbacked conch was upheld over a relatively wide range of P_{O_2}, showing that it can be considered to be an oxy-regulator. Indeed, the low $P_{O_2,crit}$ (~3 kPa) indicates that this gastropod has a high capacity to extract oxygen from the water, even at low oxygen tensions. The hypoxia tolerance of the humpbacked conch, at least in terms of $P_{O_2,crit}$, is high compared with that of some other (Rao and Devi, 1984; Kapper and Stickle, 1987), but not all (Newell et al., 1978), marine gastropods that have been investigated. The finger plough shell (*Bullia digitalis*) has a $P_{O_2,crit}$ of 5.3 kPa at a much lower temperature (10°C), which should be less demanding, and interestingly its $\dot{M}_{O_2}$ virtually ceases at 2.9 kPa (Wynberg and Brown, 1986). While the inability of the finger plough shell to take up any O_2 when the oxygen level is still ~14% of air saturation could be interpreted as a low hypoxia tolerance, it may also reflect an earlier switch to anaerobic metabolism than in other species. This response is in fact common among invertebrates (Mangum and Van Winkle, 1973), which may also relate to the diversity of anaerobic pathways adopted by this animal group, discussed above.

As expected, $P_{O_2,crit}$ increased significantly with temperature, probably due to the higher $\dot{M}_{O_2,rest}$ at 33°C compared with 28°C, and in theory a higher $P_{O_2,crit}$ should indicate lower hypoxia tolerance and thereby potentially become limiting in a warmer future. But the increase in $P_{O_2,crit}$ was in fact small (1 kPa corresponding to less than a 5% reduction), and it is questionable whether such a small difference is of functional relevance. So, it is reasonable to expect that the humpbacked conch largely retains its hypoxia tolerance at higher temperatures. Even if temperature increases in the future and hypoxic events become more frequent, the humpbacked conch seems well prepared to cope with such environmental changes. This is in contrast to its coral reef fish neighbours, which show a pronounced increase in $P_{O_2,crit}$ (23–73%) when faced with a smaller change in temperature from 29 to 32°C (Nilsson et al., 2010). Together with the high aerobic scope, the low $P_{O_2,crit}$ of the humpback conch, even at increased temperature, reveals a substantial capacity of the ventilatory and circulatory systems to take up and distribute oxygen (in addition to metabolites and CO_2) and regulate oxygen uptake according to demand.

Effect of increased temperature

When temperature was raised acutely from 28 to 33°C, $\dot{M}_{O_2,rest}$ increased with a Q_{10} of ~2, which is similar to the response observed in the apple murex (*Phyllonotus pomum*) and the fighting conch (*Phyllonotus pomum*) (Sander and Moore, 1978). Interestingly, the Q_{10} of ~2 was maintained even after snails were kept at the higher temperature for 1–3 weeks, which indicates that the humpbacked conch is either unable to compensate (at least within the time frame investigated here), i.e. down-regulate $\dot{M}_{O_2}$ when faced with an increased temperature, or that there is no benefit in doing so. While down-regulation of $\dot{M}_{O_2,rest}$ during acclimation to a higher temperature can be considered beneficial from an energy-saving perspective, this does not necessarily imply that not doing so is a disadvantage, if these snails are not energy restricted. The isolated effect of temperature on basic oxygen demands in gastropods has been investigated intensively, although the majority of the data are from cold-water or temperate species. Studies done at 20–25°C show that acclimation in the form of a compensatory down-regulation of $\dot{M}_{O_2}$ at an elevated temperature occurs in some (e.g. Newell and Pye, 1971; Newell and Kofoed, 1977; Hahn, 2005) but not all cases (e.g. Calow, 1975; Shumway and Koehn, 1982; McMahon et al., 1995). It is possible that the 1–3 weeks used in the present study was too short to induce physiological compensation, but in other studies a period of 1 week, and even as short as 2 days, has been enough to induce acclimation, i.e. a down-regulation in $\dot{M}_{O_2,rest}$ or an increase in temperature tolerance (Carlisle and Cloudsley-Thompson, 1968; Newell and Pye, 1970a,b; Hamby, 1975).

The marked increase in $\dot{M}_{O_2,rest}$ with temperature might have been expected to cause aerobic scope to decline, because $\dot{M}_{O_2,max}$ increases less than $\dot{M}_{O_2,rest}$ at high temperatures in many reef fishes (Munday et al., 2012; Rummer et al., 2014). However, there was no decline in AAS at higher temperatures. While there was a tendency for FAS to decrease with temperature, this can largely be attributed to the way FAS is calculated: if $\dot{M}_{O_2,rest}$ and $\dot{M}_{O_2,max}$ increase by the same absolute amount, say 100 mg O_2 kg^{-1} h^{-1}, the ratio between the two will decrease (e.g. 500/100=6, but 600/200=3). In contrast, AAS will be maintained under the same circumstances (e.g. 500–100=400 and 600–200=400). Correspondingly, the humpbacked conch maintained AAS even at 38°C. Arguably, AAS says quantitatively more about the functional aerobic capacity (e.g. Clark et al., 2013), as the value, at least theoretically, is a more direct determinant of available energy for processes like protein synthesis and physical activity, for which the costs are essentially temperature independent (Brett, 1979; Brett and Groves, 1979).

A similar maintenance of aerobic capacity despite large increases in temperature, up to 40–45°C, has been found in some intertidal snails (Newcombe et al., 1936; Newell, 1973; Brown and da Silva, 1983, 1984; Patnaik et al., 1985). Of course, there are differences between studies in terms of methodology, animal size and type of activity, which can influence the results. But overall it appears that many gastropods, particularly intertidal species, have the ability to maintain aerobic capacity at very high temperatures. The range of temperatures over which aerobic scope is maintained in the humpbacked conch is particularly interesting because of the relatively modest maximum temperatures it experiences in its habitat. Our temperature loggers recorded a maximal increase in water temperature of about 4–5°C, during the day, and if the expected rise in temperature due to climate change is added, the snails could experience acute exposures up to perhaps 35°C in the future. While such temperature extremes are sufficient to deplete all aerobic scope and be lethal to many reef fishes (Nilsson et al., 2009; Rummer et al., 2014), or at least reduce aerobic scope (Gardiner et al., 2010; Johansen and Jones, 2011; Donelson et al., 2012), it appears that the humpbacked conch is not challenged at these temperatures, at least not in terms of aerobic scope. It is intriguing that this subtidal snail exhibits a relatively high temperature tolerance, when it appears to inhabit an area that is much less extreme than that of truly intertidal gastropods (e.g. Lewis, 1963; Newell, 1979; Garrity, 1984). It may be that this species has

previously inhabited a more extreme environment, and it cannot be ruled out that some populations still do.

Effects of ocean acidification

From the present study it is clear that the respiratory capacity of the humpbacked conch is unaffected by exposure to elevated CO_2 at both ambient and elevated temperatures. While ocean acidification appears to have varying and in some cases no effect on metabolism (Bibby et al., 2007; Marchant et al., 2010; Melatunan et al., 2011; Wood et al., 2011; Catarino et al., 2012; McElroy et al., 2012; Manríquez et al., 2013; Matoo et al., 2013; Schalkhausser et al., 2013, 2014; Zhang et al., 2014), a range of other important effects in molluscs, both physiological (reviewed by Parker et al., 2013; Kroeker et al., 2014) and behavioural (e.g. Vargas et al., 2013; Spady et al., 2014; Watson et al., 2014), have been described. Importantly and directly related to the current study, we recently found that exposure to elevated CO_2 significantly reduced the number of individuals that jump in response to cone snail odour, while not affecting the jumping performance or aerobic capacity of snails that 'decided' to jump (Watson et al., 2014). Thus, while elevated CO_2 may not affect aerobic scope, it could directly reduce individual survivorship by altering predator avoidance behaviour. Interestingly, elevated CO_2 has also been shown to alter defence behaviour of the tropical squid *Idiosepius pygmaeus* (Spady et al., 2014) and the predator–prey interaction between the marbled cone snail and the strawberry conch (*Strombus luhuanus*) (Fields, 2013), but it is unknown whether aerobic scope of these species is also affected by elevated CO_2. These contrasting results emphasize the need to evaluate several physiological systems when attempting to predict how organisms may cope with global change. It is also important to consider the performance of different life-history stages and the potential effects on calcification and shell development (e.g. Kurihara, 2008; Kroeker et al., 2010; Byrne, 2011), as the juvenile stages of the humpbacked conch may be more sensitive and thereby limit future fitness.

Will jumping snails prevail?

The ability of the humpbacked conch to maintain aerobic scope at elevated temperatures appears sufficient not only for today's needs but also those of a warmer future. Similarly, it is also capable of maintaining its hypoxia tolerance (low $P_{O_2,crit}$) at elevated temperatures, and neither of these capacities is compromised by projected future CO_2 levels. The finding of such abilities in an animal considered to have a relatively simple circulatory system is intriguing, and how its oxygen uptake and delivery are regulated during exercise and increased temperature warrants further study. The recently discovered behavioural impairment in some individuals after elevated-CO_2 exposure (Watson et al., 2014) may still reduce the success of these snails. However, if there is a genetic component to the individual variation, CO_2-insensitive individuals may be favoured by natural selection, making this temperature-tolerant species one of the winners in a warmer acidified future.

MATERIALS AND METHODS

Animals

Humpbacked conch, *Gibberulus gibberulus gibbosus* (Röding 1798), were collected in November–December 2013 from the Lizard Island Lagoon (supplementary material Fig. S1), Great Barrier Reef, Australia (14°41′ 31.2″S 145°27′56.5″E). Collection was done by snorkelling and as the snails were mostly burrowed ∼5 cm down in the sand, they were gently exposed using a rake, and could then be collected by hand. They were transferred to an environmentally controlled flow-through aquarium facility at Lizard Island Research Station. Snails were housed in 32 l (38×28×30 cm L×W×H) white plastic containers (20–30 individuals in each, only ∼12 individuals from each container were used) supplied with a continuous flow of seawater. Oxygen levels were checked daily and remained at >95% air-saturation throughout the experiment. The snails fed on algal film, which was abundant on the surfaces of each aquarium.

Temperature and CO_2 treatment

Ninety-seven snails (wet tissue mass 0.98±0.23 g, mean±s.d.) were randomly divided into four exposure groups (two containers per group): 28°C–ambient CO_2, 28°C–elevated CO_2, 33°C–ambient CO_2 and 33°C–elevated CO_2. Temperature was monitored and controlled by an AquaMedic T-computer (AquaMedic GmbH, Bissendorf, Germany), connected to titanium heaters (AquaMedic). Water in the 33°C tanks was initially raised from 28°C over a period of 5 h. Elevated-CO_2 seawater was achieved by dosing with CO_2 to a set pH. Seawater was pumped from the ocean into two, 60 l header tanks where it was diffused with ambient air (ambient-CO_2 treatment) or CO_2 gas to achieve the desired pH (elevated-CO_2 treatment) (see Table 2 for CO_2 values obtained). A pH controller (AquaMedic) attached to the elevated-CO_2 treatment header tank maintained pH at the desired level. Seawater pH_{NBS} was recorded daily (Mettler Toledo SevenGo pH with InLab®413 SG/2m probe, Mettler-Toledo International, Inc., Columbus, OH, USA) in each aquarium and seawater CO_2 confirmed with a portable CO_2 equilibrator and infrared sensor (GMP343, Vaisala, Helsinki, Finland) (Munday et al., 2014). Water samples were analysed for total alkalinity by Gran titration (888 Titrando, Metrohm, Switzerland) to within 0.4% of certified reference material (Prof. A. Dickson, Scripps Institution of Oceanography). Carbonate chemistry parameters (Table 2) were calculated using the CO2SYS.xls workbook (Pierrot et al., 2006), selecting 'Mehrbach et al.' for constants K_1 and K_2, and 'Dickson' for K_{SO_4}. The elevated-CO_2 snails were transferred directly from ambient CO_2 to elevated CO_2 and heating of tanks to 33°C was initiated at least 1 day after transferral to an elevated-CO_2 tank. The snails were exposed to the elevated temperature and/or CO_2 conditions for at least 1 week (12±4 days, mean±s.d.) before experimentation.

During respirometry, snails were measured at their treatment temperature (28°C-acclimated snails at 28°C or 33°C-acclimated snails at 33°C) or exposed acutely to a 5°C higher temperature (28°C-acclimated snails at

Table 2. Average temperature and parameters of water chemistry

Treatment	28°C–ambient CO_2	28°C–elevated CO_2	33°C–ambient CO_2	33°C–elevated CO_2
Temperature (°C)	28.4±0.7	28.5±0.7	32.6±0.6	32.6±0.4
Salinity (ppt)	35.5±0.1	35.5±0.1	35.5±0.1	35.5±0.1
pH_{NBS}	8.17±0.03	7.87±0.02	8.14±0.02	7.86±0.02
Total alkalinity (μmol kg^{-1} SW)	2288.0±33.9	2294.3±9.4	2288.0±33.9	2294.3±9.4
P_{CO_2} (μatm)	416.7±33.0	955.1±57.3	453.5±26.3	986.5±54.1
P_{CO_2} (kPa)	0.0422±0.0033	0.0968±0.0058	0.0460±0.0027	0.1000±0.0055
Ω_{Ca}	5.53±0.27	3.15±0.14	5.78±0.24	3.42±0.17
Ω_{Ar}	3.70±0.18	2.10±0.10	3.91±0.17	2.31±0.12

Data are means±s.d. of the daily measurements from the entire experiment period (2–28 December 2013). Ω, saturation state of seawater with respect to aragonite (Ω_{Ar}) or calcite (Ω_{Ca}).

33°C, 33°C-acclimated snails at 38°C). The CO_2 level during measurement was always the same as in the respective holding tanks (ambient-CO_2 snails were only tested at ambient CO_2; elevated-CO_2 snails were only tested at elevated CO_2).

Respirometry

Aerobic scope

Obviously, to measure aerobic scope it is necessary to estimate minimum oxygen uptake ($\dot{M}_{O_2,min}$) and maximum oxygen uptake ($\dot{M}_{O_2,max}$), but is difficult to be certain that an animal is in either of these states. $\dot{M}_{O_2,min}$ can, however, be approximated by measurement of resting oxygen uptake ($\dot{M}_{O_2,rest}$; a fasting unstressed animal showing minimal movement) and $\dot{M}_{O_2,max}$ can be estimated by measuring $\dot{M}_{O_2}$ in a state of maximum inducible activity. The aerobic scope calculated from these measurements will be comparable between different treatments if all individuals are measured in the same states, even though the absolute values of AAS and FAS might be underestimated.

Protocol

Individual snails from the treatment groups were transferred to four identical respirometers (136 ml) that could be used simultaneously, all submerged in a larger flow-through aquarium with controlled temperature and CO_2 level. The respirometers were supplied with water from this aquarium. Each respirometer contained a small magnetic propeller (driven by magnetic stirrers outside the aquarium). This ensured proper mixing of the water inside the respirometers. Immediately after introduction into the respirometer, jumping was induced by injection of 50 ml of cone snail-scented water into the respirometer (see supplementary material Movie 1). During injection, excess water was expelled through a small outlet (0.5 cm in diameter, 3 cm high) on top of the respirometer and the respirometer water volume therefore remained the same. The cone snail-scented water was obtained by placing one cone snail in 2 l of water (conditioned to the proper temperature and CO_2 level) for 10–20 min. Initial tests showed that injection of cone odour into an empty respirometer in itself did not cause an increase in background $\dot{M}_{O_2}$ (data not shown, N=8, Kruskal–Wallis ANOVA, P=0.6095). P_{O_2} in the respirometer and temperature in the water were recorded 20 times per minute with a 4-channel optical oxygen meter (FireStingO_2, PyroScience GmbH, Aachen, Germany) using the Firesting Logger software, while the number of jumps and the time until jumping ceased were observed visually and recorded. When a snail had not jumped for 3 min, the respirometer water was exchanged to remove cone snail odour (as preliminary trials revealed that the snails would otherwise resume their jumping activity after a while) and to restore the oxygen level. The snail was then left in the respirometer and oxygen consumption was measured with intermittent-flow respirometry for an additional 16–20 h. During this time the snails recovered from the exercise and entered a resting state of oxygen consumption. During intermittent-flow respirometry, the respirometer was flushed for 15 min every hour by a small pump controlled by an on–off timer (Steffensen et al., 1984; Steffensen, 1989). After the measurements, the snail was removed from the respirometer and returned to its holding tank. All snails recovered from the exercise and respirometry experiment, including those that had been acutely exposed to high temperature. Background oxygen consumption in the respirometers was measured both in new treatment seawater before introducing a snail and after taking out the snail at the end of the trial. After recovery, snails were submerged for 5 min (or until responses to tactile stimuli subsided) in crushed ice, after which the shell was cracked using a vice and all shell peeled away from the soft tissue. Wet tissue mass was then measured on a precision balance.

Calculations

Raw data (P_{O_2} versus time) were exported to LabChart® Reader 8.0 (ADInstruments Ltd, Oxford, UK) to determine the slope ($\Delta P_{O_2}/\Delta t$, where ΔP_{O_2} is the decrease in oxygen partial pressure) for each closed interval in the respirometer. This was done by marking each interval, using the built-in 'calculate average slope' and 'copy to data-pad' function. This slope was then used to calculate $\dot{M}_{O_2}$ using Eqn 1:

$$\dot{M}_{O_2} = \frac{|\Delta P_{O_2}/\Delta t| \times \beta_{w,O_2} \times V_{sys}}{M_b}, \quad (1)$$

where β_{w,O_2} is the capacitance coefficient for oxygen in water (dependent on temperature and salinity), V_{sys} is the volume of water in the respirometers and M_b is wet tissue body mass. All $\dot{M}_{O_2}$ points were corrected for the background $\dot{M}_{O_2}$ in the respirometer. As background $\dot{M}_{O_2}$ could only be measured before and after, the background in between was estimated by linear regression. The average background $\dot{M}_{O_2}$ was 16±7% (mean±s.d.) of the average $\dot{M}_{O_2}$. $\dot{M}_{O_2,rest}$ was determined as the lowest 10th percentile (Chabot and Claireaux, 2008) to minimize the effect of spontaneous activity while also taking into account the effect of measurement error. Snails that did not appear to enter a resting state during the measurement were excluded from further calculations. The temperature coefficient Q_{10} (the increase in $\dot{M}_{O_2}$ over a 10°C increase in temperature) was calculated according to Eqn 2:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2-T_1)}, \quad (2)$$

where R_1 and R_2 are the $\dot{M}_{O_2}$ at temperatures T_1 and T_2. $\dot{M}_{O_2,max}$ was calculated from the slope during jumping (first 3–5 min of the experiment). The proportional decrease in P_{O_2} during this interval was 5.4±3.0% (mean±s.d.), dependent on size and activity, compared with an overall noise level of 0.2±0.1% (mean±s.d.) for the P_{O_2} signal, giving lines with an R^2 of 98.6±1.4% (mean±s.d.). Furthermore, snails that had a low $\dot{M}_{O_2,max}$ in combination with low jumping activity were excluded from further calculations, as it could not be assumed that the measured $\dot{M}_{O_2}$ was close to $\dot{M}_{O_2,max}$. AAS was calculated for the individuals where a reliable estimate was obtained for both $\dot{M}_{O_2,rest}$ and $\dot{M}_{O_2,max}$ as $\dot{M}_{O_2,max}-\dot{M}_{O_2,rest}$. FAS was calculated as $\dot{M}_{O_2,max}/\dot{M}_{O_2,rest}$. The rate of jumping was calculated by dividing the number of jumps performed with the time period during which jumping occurred. The amount of oxygen consumed per jump (cost per jump) was calculated using Eqn 3:

$$\text{Cost per jump} = \frac{(\dot{M}_{O_2,max} - \dot{M}_{O_2,rest}) \times \text{Time spent jumping}}{\text{Total number of jumps.}} \quad (3)$$

To examine the relationship between $\dot{M}_{O_2}$ and jumping, the total amount of oxygen consumed during jumping was calculated by multiplying the cost per jump (Eqn 3) by the total number of jumps, and expressing it as a function of the total number of jumps. Lastly, excess EPOC was calculated for each snail following the method commonly used for fish (Scarabello et al., 1991; Lee et al., 2003). Briefly, this was done by first subtracting $\dot{M}_{O_2,rest}$ from all points and then excluding 'high' values (i.e. values should be decreasing continuously from maximum as sudden increases are probably due to activity and not EPOC). An exponential decay curve was then fitted to the remaining $\dot{M}_{O_2}$ data, and the area under the curve was calculated as the defined integral from the time the trial began to when $\dot{M}_{O_2,rest}$ was reached, giving EPOC in mg O_2 kg^{-1}. Curve fitting was done in SigmaPlot® 12.5 (Systat Software, Inc., Washington, IL, USA) while the integration was performed in LabChart® Reader 8.0 (ADInstruments).

$P_{O_2,crit}$

A subset of 8–10 snails from each of the four treatment groups that had been used to measure aerobic performance as described above were also used for measurement of $P_{O_2,crit}$. Measurements were only done on snails that had been kept at their treatment temperature during the preceding intermittent-flow respirometry, i.e. not on the snails that had been exposed to acute-33°C or acute-38°C. Snails were allowed to recover for 24 h in their holding tank before being transferred to a respirometer (230 ml) without flow (closed respirometry; Nilsson et al., 2010). This ensured that they had been allowed to recover for approximately 40 h in total after jumping (15+24 h), or even up to 50 h, taking into account the time it took for the water in the closed respirometer to become hypoxic. The P_{O_2} in the respirometer was measured until it approached zero, which took 11±3 h (mean±s.d.), using a galvanometric oxygen probe (OXI 340i, WTW GmbH, Weilheim, Germany) equipped with a small magnetic propeller driven by a magnetic stirrer placed outside the chamber, ensuring proper mixing of the water in the respirometer. Data were recorded with Power Lab 4/20 using Chart v. 5.4.2 software (ADInstruments). Data were exported to LabChart®

Reader 8.0 (ADInstruments) to determine $\Delta P_{O_2}/\Delta t$ as described above, with the addition that the average P_{O_2}, over which the slope was calculated, was also reported. Because the data trace consisted of a gradually declining P_{O_2} and not regularly spaced intervals, the P_{O_2} value for each $\dot{M}_{O_2}$ value was not always the same between individuals, and data are therefore presented as means±s.e.m. for P_{O_2}. Note that the data in Fig. 4A are presented for graphical visualization only. The $P_{O_2,crit}$ was determined for each individual snail as the P_{O_2} at the intersection between a linear regression line fitted to the points at the low-O_2 end of the plot where $\dot{M}_{O_2}$ decreased with falling P_{O_2} and a horizontal line representing the individual $\dot{M}_{O_2,rest}$ measured during the preceding intermittent-flow respirometry (i.e. we did not derive $\dot{M}_{O_2,rest}$ values from the closed respirometry) (Ultsch et al., 1980; Berschick et al., 1987; Nilsson et al., 2010). By using this $\dot{M}_{O_2,rest}$ value measured after the long habituation, potential overestimation of this value due to increased activity or stress during the first hours in the closed respirometer was avoided (Lefevre et al., 2011, 2014b).

Temperature measurements in the field

Temperature and light intensity loggers (HOBO® Pendant Temp/Light Logger – 64K Samples, Onset Computer Corporation, Bourne, MA, USA) were placed at three different sites at the location where snails were collected (supplementary material Fig. S1A). Two loggers were placed 5 cm into the sand, 2–3 m apart at a 90 deg angle from the beach (approximately 30 cm difference in water depth) (supplementary material Fig. S1B, site A_S and B_S). A third logger was placed in the water column 5 cm above the sand (site B_W). These sites represent the boundaries between which most snails were found. Data were logged every minute for 4 days and then exported to Excel using HOBOware Pro 3.

Statistics

Data were analysed in GraphPad Prism® 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). Data were tested for normality (Shapiro–Wilk's test) and variance homogeneity (Bartlett's test), and transformed if necessary (natural logarithm). The effect of time and CO_2 on $\dot{M}_{O_2}$ after jumping was analysed using a repeated-measures two-way ANOVA, followed by a Šídák's multiple comparison test against $\dot{M}_{O_2,rest}$. A linear regression analysis was used to examine the relationship between the total number of jumps and the total amount of oxygen consumed during jumping. A one-way ANOVA followed by a Šídák's multiple comparison was used to detect differences between the 12 physiologically relevant treatment pairs. The effect of temperature and CO_2 on $P_{O_2,crit}$ was analysed with a two-way ANOVA, followed by a Šídák's multiple comparison test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.L., G.E.N., P.L.M. and S.-A.W. conceived the study. S.L. and G.E.N. carried out the experiments. S.L. analysed the data. P.L.M. and S.-A.W. assisted with animal collection, setting up the CO_2 system and analysing water chemistry. S.L., G.E.N., P. L.M. and S.-A.W. wrote and revised the manuscript.

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Supplementary material

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