

## RESEARCH ARTICLE

# Aerobic scope does not predict the performance of a tropical eurythermal fish at elevated temperatures

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## ABSTRACT

Climate warming is predicted to negatively impact fish populations through impairment of oxygen transport systems when temperatures exceed those which are optimal for aerobic scope (AS). This concept of oxygen- and capacity-limited thermal tolerance (OCLTT) is rapidly gaining popularity within climate change research and has been applied to several fish species. Here, we evaluated the relevance of aerobic performance of juvenile barramundi (*Lates calcarifer*) in the context of thermal preference and tolerance by (1) measuring standard and maximum metabolic rates (SMR and MMR, respectively) and AS of fish acclimated to 29°C and acutely exposed to temperatures from 23 to 38°C, (2) allowing the fish to behaviourally select a preferred temperature between 29 and 38°C, and (3) quantifying alterations to AS after 5 weeks of acclimation to 29 and 38°C. SMR and MMR both increased continuously with temperature in acutely exposed fish, but the increase was greater for MMR such that AS was highest at 38°C, a temperature approaching the upper lethal limit (40–41°C). Despite 38°C eliciting maximum AS, when given the opportunity the fish selected a median temperature of 31.7±0.5°C and spent only 10±3% of their time at temperatures >36°C. Following acclimation to 38°C, AS measured at 38°C was decreased to the same level as 29°C-acclimated fish measured at 29°C, suggesting that AS may be dynamically modulated independent of temperature to accommodate the requirements of daily life. Together, these results reveal limited power of the OCLTT hypothesis in predicting optimal temperatures and effects of climate warming on juvenile barramundi.

**KEY WORDS:** Barramundi, Climate change, *Lates calcarifer*, Metabolic rate, Oxygen- and capacity-limited thermal tolerance (OCLTT), Oxygen consumption rate

## INTRODUCTION

The progressive warming of aquatic ecosystems is of critical conservation concern for fish as it has been associated with shifts in phenology, distribution and abundance (e.g. Perry et al., 2005; Pörtner and Knust, 2007; Martins et al., 2011), yet the temperature-sensitive mechanisms driving these phenomena remain speculative. A leading hypothesis is that these population-level changes result from a decrease in aerobic metabolic performance of fish with increasing temperature, caused by a gradual decline in the capacity of the ventilatory and circulatory systems to deliver oxygen to the respiring tissues (i.e. oxygen- and capacity-limited thermal tolerance, OCLTT) (Pörtner and Knust, 2007; Pörtner and Farrell,

2008; Martins et al., 2011). The mismatch between oxygen supply and demand manifests as a decrease in aerobic scope (the degree to which oxygen consumption rate,  $\dot{M}_{O_2}$ , can be increased above resting levels) at either side of an optimal temperature for aerobic scope ( $T_{opt,AS}$ ) set by lower and upper pejus temperatures ( $T_p$ ) (Pörtner and Farrell, 2008). Consequently, overall animal performance across temperature is proposed to resemble a bell-shaped curve with a peak at  $T_{opt,AS}$ , the temperature where critical fitness-related factors such as locomotion, growth and reproduction are thought to be optimised (Frederich and Pörtner, 2000; Pörtner, 2002; Wang and Overgaard, 2007; Pörtner and Farrell, 2008; Pörtner, 2010; Pörtner, 2012). This bell-shaped performance curve suggested for aquatic ectotherms contrasts with thermal performance curves suggested for many terrestrial ectotherms that are highly left-skewed (i.e. right-biased) (Gilchrist, 1995; Martin and Huey, 2008).

The foundation for much of the OCLTT concept stems from studies of species inhabiting open oceans (Frederich and Pörtner, 2000; Pörtner and Knust, 2007) where temperature fluctuations are dampened by the water masses and warming is gradual. In contrast, the temperature of coastal and estuarine waters can vary markedly over short temporal scales of hours to days, which is likely to be exacerbated in the event of future increases in storm intensity and daily temperature extremes (Trenberth, 2012). Because these acute environmental changes have the capacity to act rapidly and at the individual level without the opportunity for prior acclimation, coastal species that live in dynamic environments provide interesting models for investigating the effects of acute thermal exposure on animal performance and the OCLTT concept. Even so, the degree to which acute versus chronic thermal exposure influences the aerobic performance of fish in general is not well understood and requires significantly more attention.

To shed light on these knowledge gaps, the present study used juvenile barramundi (*Lates calcarifer* Bloch 1790) from central-eastern Queensland, Australia, as a model organism to examine aerobic performance and thermal preferences in the framework of the OCLTT hypothesis. In tropical Australia, juvenile barramundi inhabit estuaries, coastal swamps and tidal creeks (Pusey et al., 2004) where temperatures can range from 23 to 36°C during summer months (Russell and Garrett, 1985; Loong et al., 2005). Here, fluctuations in water temperature of 10°C or more occur at a rate of up to 2°C h<sup>-1</sup> (Loong et al., 2005), which is likely to intensify if predictions of more extreme climatic events hold true (Lima and Wetthey, 2012; Trenberth, 2012). Recent studies on juvenile barramundi across a temperature range of 21–39°C suggest that growth performance is optimal around 31°C (Katersky and Carter, 2007; Bermudes et al., 2010; Glencross and Bermudes, 2012) and remains high (≥90% of maximum) over a wide range of temperatures (~27–36°C) before declining precipitously at the extremes (Katersky and Carter, 2007; Bermudes et al., 2010). In addition, locomotor capacity (critical swimming speed,  $U_{crit}$ ) in juvenile barramundi from Australia is reported to be thermally

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**List of symbols and abbreviations**

AS	aerobic scope
FAS	factorial aerobic scope
MMR	maximum metabolic rate
$\dot{M}_{O_2}$	oxygen consumption rate
OCLTT	oxygen- and capacity-limited thermal tolerance
SMR	standard metabolic rate
$T_{opt,AS}$	optimum temperature for aerobic scope
$T_p$	pejus temperature

independent from 25 to 35°C ('northern' population from Darwin, Northern Territory) or optimised around 30°C ('southern' population from Bowen, Queensland) (Edmunds et al., 2010). This apparent thermal preference around 30–31°C seems to be quite well established, as commercial aquaculture industries around Australia generally maintain their barramundi around this temperature range and report growth to be optimised between 28 and 32°C (Schipf et al., 2007). Based on this existing information, it was hypothesised in the present study that  $T_{opt,AS}$  would occur around 30–31°C and that fish would preferentially select temperatures around this range. Furthermore, we expected that acute and chronic exposure to temperatures approaching the upper critical temperature of the species (i.e. 40–41°C) would cause AS to approach zero as predicted from a bell-shaped performance curve. It was anticipated that the results of this study would provide insight into the future performance, distribution and abundance of this species, as has been reported for other species using the OCLTT framework (e.g. Pörtner and Knust, 2007; Farrell et al., 2008; Nilsson et al., 2009; Eliason et al., 2011).

**RESULTS**

For the acutely exposed fish,  $\dot{M}_{O_2}$  stabilised quickly at each temperature and both SMR and MMR increased exponentially (from 23 to 38°C) with significant differences between all temperatures (one-way repeated measures ANOVA: SMR,  $P<0.001$ ; MMR,  $P<0.042$ ) (Fig. 1A; Fig. 2). Importantly, the follow-up assessments of SMR and MMR at 29°C were not significantly different from the initial trials at 29°C (paired  $t$ -test: SMR,  $P=0.291$ ; MMR,  $P=0.264$ ), confirming that repeated exercise at a range of temperatures did not compromise aerobic performance.  $Q_{10}$  coefficients between 23 and 38°C were  $2.32\pm0.08$  and  $1.71\pm0.05$  for SMR and MMR, respectively. AS also increased exponentially with temperature ( $Q_{10}=1.56\pm0.06$ ; Fig. 1B) with significant differences between all temperatures from 29 to 38°C (one-way repeated measures ANOVA,  $P<0.012$ ) but not between 23 and 29°C (one-way repeated measures ANOVA,  $P=0.175$ ). Factorial aerobic scope (FAS) tended to decrease with temperature (Fig. 1C) but was only significantly different at 23°C in comparison with other temperatures (one-way repeated measures ANOVA,  $P<0.001$  in all cases).

The median temperature selected by the fish in the thermal preference experiment was  $31.7\pm0.5^\circ\text{C}$  with the first and third quartile being  $31.0\pm0.3$  and  $33.4\pm0.7^\circ\text{C}$ , respectively. The mean temperature was  $32.2\pm0.4^\circ\text{C}$  and the majority of time was spent at  $31.2\pm0.4^\circ\text{C}$  (the modal temperature). Data for each individual fish are listed in Table 1 and highlight that no individual fish selected a median, mean or modal temperature higher than  $34.0^\circ\text{C}$ . Frequency histograms of the thermal record for individual fish (Fig. 3A) and for all fish combined (Fig. 3B) clearly show a preference for cooler temperatures, with  $77\pm7\%$  of time spent below  $34^\circ\text{C}$  and only  $10\pm3\%$  of time spent above  $36^\circ\text{C}$  (Fig. 3B).

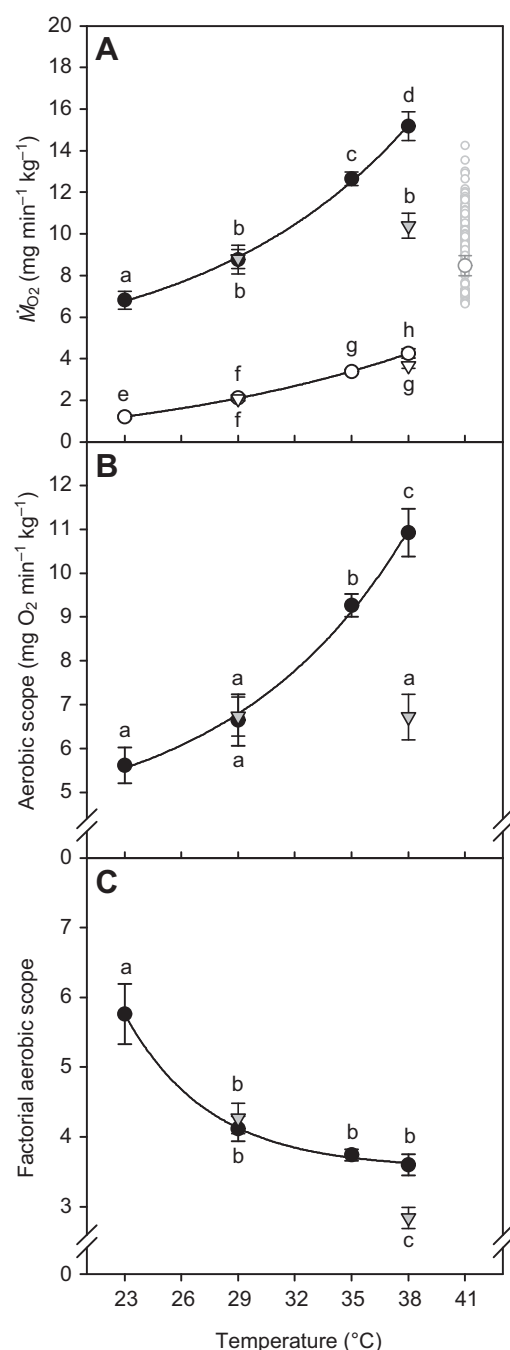
SMR and MMR data obtained at 29°C following an additional 5 weeks of acclimation to 29°C were indistinguishable from the data

obtained from the initial trials at 29°C ( $t$ -test: SMR,  $P=0.779$ ; MMR,  $P=0.974$ ; Fig. 1), indicating no influence of longer-term captivity or increased body mass on mass-specific  $\dot{M}_{O_2}$ . However, fish acclimated to 38°C for 5 weeks and tested at this temperature showed a 14% reduction in SMR, a 32% reduction in MMR ( $t$ -test: SMR,  $P=0.046$ ; MMR,  $P<0.001$ ; Fig. 1A), a 39% reduction in AS ( $t$ -test,  $P<0.001$ ; Fig. 1B), and a 21% reduction in FAS ( $t$ -test,  $P=0.001$ ; Fig. 1C) when compared with the fish acutely exposed to 38°C in initial trials. Interestingly, the AS of 38°C-acclimated fish at 38°C was essentially identical to the AS of 29°C-acclimated fish at 29°C, suggesting full thermal compensation of AS upon acclimation to 38°C.

**DISCUSSION**

This study shows a continual increase in MMR and AS of juvenile barramundi as temperatures acutely approach lethal limits, resulting in a  $T_{opt,AS}$  of 38°C for acutely exposed fish. As a temperature of 38°C is unlikely to be encountered in the life cycle of most barramundi (Pusey et al., 2004), and the fish were clearly troubled when temperature was increased further to 41°C, this study highlights that AS can be maximal at temperatures at the extreme high end of ecological relevance. This is supported by results in a pioneering study on bullhead (*Ameiurus nebulosus*), where AS increased up to the 'upper incipient lethal temperature' of  $\sim 37^\circ\text{C}$  (Fry, 1947). Similarly, a study on pink salmon (*Oncorhynchus gorbuscha*) documented a  $T_{opt,AS}$  of 21°C for acutely exposed individuals, which is at the uppermost end of the temperature range experienced by the species at any point in its life cycle (Clark et al., 2011), and a temperature that causes death within days when fish are chronically exposed in captivity (Jeffries et al., 2012). Such left-skewed AS curves differ from the bell-shaped change in AS with temperature that has been suggested for aquatic ectotherms (Frederich and Pörtner, 2000; Pörtner and Farrell, 2008; Pörtner, 2010; Pörtner, 2012). The acute rate of temperature change used in the present study ( $2^\circ\text{C h}^{-1}$ ) is consistent with the extreme rates of temperature change measured in juvenile barramundi habitats of tropical Australia (Loong et al., 2005). Notably, this is the same rate of temperature increase used by Frederich and Pörtner (Frederich and Pörtner, 2000) in their study on hemolymph oxygenation and cardiac performance of the spider crab (*Maja squinado*), which has contributed heavily to the foundation for the OCLTT concept.

The OCLTT concept assumes that AS of water-breathers becomes limited due to a failure of MMR to increase at the same pace as SMR as temperatures exceed  $T_{opt,AS}$  (Farrell et al., 2009), thus creating a bell-shaped performance window. This failure is speculated to lie with the heart, where a limit on maximum heart rate causes a cascade of events leading to a perfusion limitation to swimming muscles and a resultant acidemia and hyperkalemia of venous blood as muscles are forced to work glycolytically (Farrell et al., 2009). This, in turn, is thought to further impair heart function and cause a gradual demise in oxygen transport capacity as warming continues. The OCLTT concept has been applied to several species of fish, both temperate and tropical, with the latter proposed to be especially vulnerable to climate warming due to a present day existence at the upper edge of their aerobic performance window (Munday et al., 2009; Nilsson et al., 2009; Gardiner et al., 2010). Results from the present study do not support such a limitation in AS for juvenile barramundi, and they contrast with the idea that high temperature tolerance is governed by oxygen transport. The impressive thermal tolerance range for barramundi corresponds well with the ecology of this eurythermal species as adults spawn in coastal areas and the juveniles move into wetland and estuarine



**Fig. 1. Aerobic metabolic performance (means  $\pm$  s.e.m.) of acutely exposed (circles;  $N=7$  or  $8$ ) and thermally acclimated (triangles;  $N=4$  for  $29^\circ\text{C}$  and  $N=8$  for  $38^\circ\text{C}$ ) juvenile barramundi at different temperatures.** (A) Standard metabolic rate (SMR; white circles, white triangles) and maximum metabolic rate (MMR; black circles, grey triangles). Note that  $\dot{M}_{O_2}$  at  $41^\circ\text{C}$  is included for comparative purposes only, as fish lost equilibrium after 2.5 h at this temperature and therefore the obtained  $\dot{M}_{O_2}$  does not represent either SMR or MMR. Data at  $41^\circ\text{C}$  are all  $\dot{M}_{O_2}$  measurements for all fish (small grey circles) as well as the overall mean  $\pm$  s.e.m. (larger grey circle) of the lowest three  $\dot{M}_{O_2}$  measurements for each fish during the 2.5 h period. (B) Aerobic scope (AS; black circles, grey triangles); (C) factorial aerobic scope (FAS; black circles, grey triangles). Different letters indicate significant differences between measurements. For the acutely exposed fish, SMR and MMR increased exponentially with ambient temperature ( $T$ ) ( $23\text{--}38^\circ\text{C}$ ) according to  $\text{SMR} = -0.7769(1.7468) + 0.4791(0.7648)e^{[0.0619(0.0333)T]}$  ( $r^2=0.904$ ,  $P<0.0001$ ) and  $\text{MMR} = 3.8207(3.1769) + 0.3704(0.7477)e^{[0.0902(0.0465)T]}$  ( $r^2=0.840$ ,  $P<0.0001$ ); AS increased with  $T$  up to  $38^\circ\text{C}$  according to  $\text{AS} = 4.1405(2.0314) + 0.1254(0.3334)e^{[0.1052(0.0631)T]}$  ( $r^2=0.751$ ,  $P<0.0001$ ); and FAS decreased with  $T$  according to  $\text{FAS} = 3.5502(0.2996) + 373.0187(965.1781)e^{[-0.2230(0.1164)T]}$  ( $r^2=0.664$ ,  $P<0.0001$ ). Values in parentheses in regression equations are standard errors.

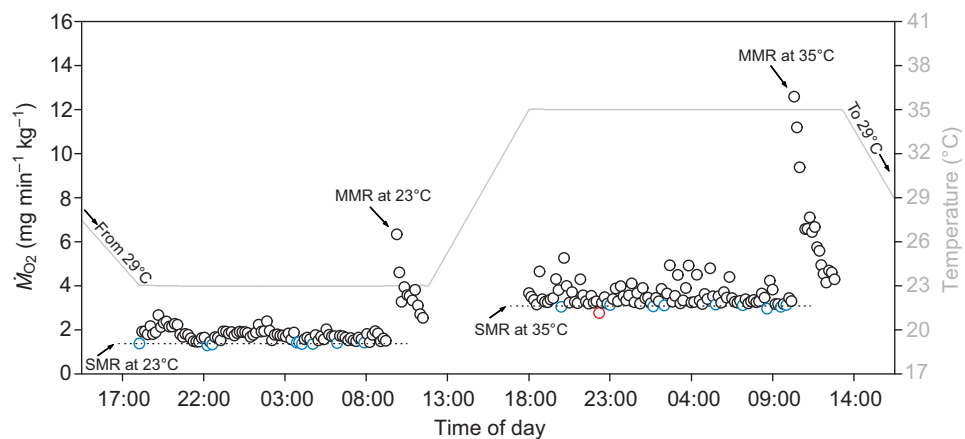
evidence of cardiorespiratory failure at temperatures immediately below the lethal limit ( $41\text{--}42^\circ\text{C}$ ) (Overgaard et al., 2012). This contrasts with the notion that the OCLTT concept should also apply to air-breathers (Pörtner, 2002) and supports the critical view on the OCLTT hypothesis recently put forward by Clark et al. (Clark et al., 2013).

The chase protocol used in the present study was chosen as the most suitable to elicit MMR in barramundi. It could be argued that this technique would induce a substantial anaerobic metabolic rate and that the acidosis of the blood would thus affect cardiac performance and the MMR measurements (Farrell, 2007; Farrell et al., 2009). However, had this potential issue caused an underestimation of MMR, this effect should either have been independent of temperature (Brett, 1964; Beamish, 1978) or greatest at the higher temperatures (Farrell, 2007), meaning that MMR (and thereby AS) should have been equally underestimated at all temperatures or even greater than what we measured at the highest temperatures. This would not change our findings of a continual increase in MMR up to  $38^\circ\text{C}$  in fish undergoing acute thermal exposures and, if anything, it would further strengthen our conclusions. It is notable that significant anaerobic metabolism also occurs during standard  $U_{\text{crit}}$  protocols (Brett, 1964; Farrell, 2007; Svendsen et al., 2010), and so the same arguments may apply. Nevertheless, identifying the shape, vertical and horizontal positions of AS versus temperature curves when MMR is obtained via different techniques (e.g. chase versus swimming respirometry) would be an interesting future endeavour in suitable species. Moreover, there is some evidence for differential performances between populations of the same species, such as an apparent difference in the thermal sensitivity of  $U_{\text{crit}}$  between two geographically separated populations of barramundi exposed to different annual temperature regimes (Edmunds et al., 2010).

Measurements of AS are recurrently being used to evaluate the impacts of aquatic warming on fish by assuming that  $T_{\text{opt,AS}}$  represents the preferred temperature of the species (e.g. Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Farrell, 2009; Munday et al., 2009; Nilsson et al., 2009; Donelson et al., 2012). Given the  $T_{\text{opt,AS}}$  of  $38^\circ\text{C}$  in  $29^\circ\text{C}$ -acclimated, acutely exposed juvenile barramundi from the present study, it may be predicted based on the OCLTT hypothesis that individuals would select  $38^\circ\text{C}$  in order to maximise AS. However, when barramundi were given the opportunity to select temperatures between  $29$  and  $38^\circ\text{C}$  the majority of time was spent

nurseries where they typically experience widely fluctuating temperatures across days and seasons. Consistent with our findings, a study on Murray cod (*Maccullochella peelii peelii*) from south-eastern Australia revealed that a continual increase in AS with temperature (from  $14$  to  $29^\circ\text{C}$ ) was accompanied by a consistent rise in heart rate scope (Clark et al., 2005). Recent research on a eurythermal crustacean, the green crab (*Carcinus maenas*), showed that cardiac output remained unaffected by acute heat stress as temperature was increased from  $10$  to  $25^\circ\text{C}$  at a rate of  $1^\circ\text{C h}^{-1}$  (Giomi and Pörtner, 2013). Taken together, these results from water-breathers resemble those recently reported for the air-breathing and semi-terrestrial cane toad (*Rhinella marina*) (Seebacher and Franklin, 2011; Overgaard et al., 2012). In this amphibian, AS was found to increase with temperature (from  $10$  to  $40^\circ\text{C}$ ) with no





**Fig. 2. Representative segment of the experimental protocol to obtain  $\dot{M}_{O_2}$  data for the barramundi.** The example is for a 25.2 g fish and shows how SMR was obtained over 15.5 h at 23°C after which the fish was removed from the respirometer, chased, and immediately placed back into the respirometer to obtain MMR at 23°C. After a 1.6 h recovery period inside the respirometer, temperature was increased to 35°C at a rate of 2°C h<sup>-1</sup> and the fish was left for 17 h for measurements of SMR once 35°C was reached. Upon completion of SMR measurements, the fish was chased at 35°C to obtain MMR at this temperature and was again left to recover before temperature was changed to 29°C. This example covers the greatest temperature change (12°C) experienced by the acutely exposed fish. The blue and red data points are the lowest 10% of the  $\dot{M}_{O_2}$  measurements for each temperature during the resting periods. The red circle denotes an outlier and the blue circles indicate the data points from which SMR was calculated for each temperature (see Materials and methods).

at 31–32°C and only 10% of time was spent at temperatures above 36°C. The preference for 31–32°C corresponds well with previous studies reporting growth performance and locomotion to be optimised around these temperatures (Katersky and Carter, 2007; Bermudes et al., 2010; Edmunds et al., 2010; Glencross and Bermudes, 2012).

The vast majority of studies investigating the OCLTT hypothesis have used relatively acute thermal exposures (e.g. Frederich and Pörtner, 2000; Sartoris et al., 2003; Lannig et al., 2004; Steinhausen et al., 2008; Farrell, 2009; Pörtner, 2010; Clark et al., 2011; Giomi and Pörtner, 2013). However, more chronic thermal exposures are arguably more relevant from a climate change perspective, especially if examining species that do not naturally experience broad thermal ranges over small temporal scales. Acclimating juvenile barramundi to 38°C for 5 weeks caused a 14% reduction in SMR at 38°C compared with 29°C-acclimated individuals acutely exposed to 38°C. Such a reduction in the basic cost of living of warm-acclimated ectotherms is to be expected (Seebacher et al., 2010; Pörtner, 2012) and is thought to be achieved by down-

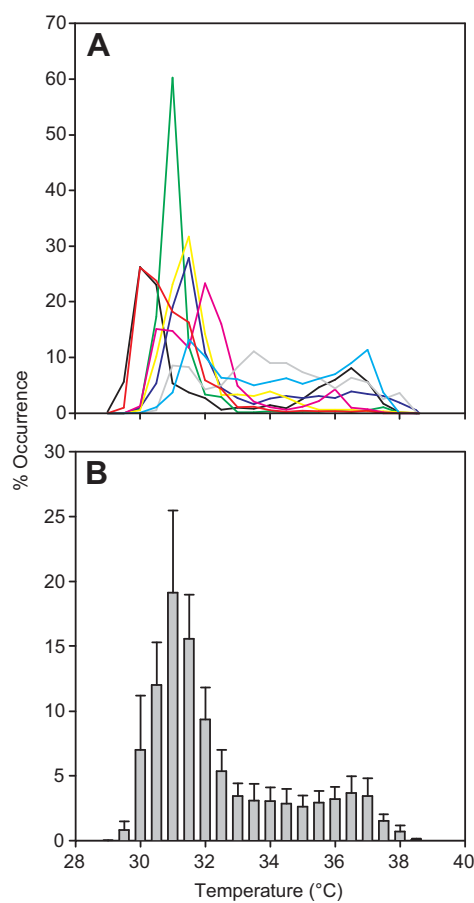
regulation of mitochondrial function (change in structure and/or density) and through changes in cell membrane structure and membrane fluidity (membrane homeoviscous adaptation) (Hazel, 1995; Seebacher et al., 2010). Although increased stress cannot be dismissed as an alternative explanation for the higher SMR of acutely exposed fish at 38°C, the concomitant 32% reduction in MMR of 38°C-acclimated barramundi supports the notion of structural changes as the driver for lowering SMR. Indeed, it is likely that reducing the metabolic machinery to conserve energy at rest comes at the price of a reduction in peak performance. The consequent decrease in AS to the same value as for 29°C-acclimated fish at 29°C suggests that AS may be modulated dynamically with the thermal environment to satisfy the requirements of daily life. Indeed, while the cardiorespiratory system of barramundi is certainly capable of much higher AS, as exemplified in 29°C-acclimated fish acutely exposed to 38°C, it is likely to be expensive and undesirable to maintain the capacity for such high performance when not regularly required for daily activities. Notably, studies maintaining juvenile barramundi at temperatures up to 38 or 39°C show a significant decrease in growth rate and feed efficiency at these high temperatures yet without any change in survival (Katersky and Carter, 2005; Katersky and Carter, 2007; Bermudes et al., 2010). Combined with our results, these data indicate that growth and feed efficiency of juvenile barramundi are not causally linked with the OCLTT hypothesis as the AS of 38°C-acclimated barramundi at 38°C was no different from that of 29°C-acclimated individuals at 29°C. Thus, despite the capacity for very high AS at 38°C, this temperature is likely to substantially impair growth rate and efficiency of juvenile barramundi, which may negatively affect survival and fitness in the natural environment due to size-selective mortality (Sogard, 1997).

The larger AS of acutely exposed over thermally acclimated barramundi contrasts slightly with data for the eurythermal killifish (*Fundulus heteroclitus*) where AS tended to be larger in thermally acclimated over acutely exposed fish (Healy and Schulte, 2012). It should be noted, however, that AS for the killifish was calculated from routine metabolic rate (rather than SMR), which may have differed between acclimated and acutely exposed fish and possibly

**Table 1. Temperature preference data for each individual fish obtained in a two-compartment figure-of-8-shaped shuttle tank with 29 and 38°C available at the extremes**

Fish	Temperature (°C)		
	Median (Q1, Q3)	Mean	Mode
1	30.4 (30.0, 35.1)	32.0	30.0
2	30.8 (30.6, 31.0)	31.0	30.6
3	31.5 (31.0, 34.0)	32.6	31.1
4	31.3 (30.9, 31.9)	31.7	31.1
5	31.8 (30.9, 32.4)	31.9	31.9
6	33.8 (32.5, 35.4)	33.9	33.5
7	34.0 (31.9, 36.0)	34.0	31.4
8	30.5 (30.0, 31.3)	30.8	29.9
Mean ± s.e.m.	31.7±0.5 (31.0±0.3, 33.4±0.7)	32.2±0.4	31.2±0.4

Data were collected over ~56 h after an initial settling period of 12 h inside the shuttle tank. Q1 and Q3 represent the first and third quartiles, respectively.



**Fig. 3. Distribution of temperatures experienced by 29°C-acclimated juvenile barramundi ( $N=8$ ) in the temperature preference experiment.**

Data for each individual were collected over ~56 h and are presented here in 0.5°C increments as (A) line plots for each individual fish and (B) a frequency histogram with bars showing mean  $\pm$  s.e.m. per cent occurrence for all fish at a given temperature.

introduced some bias to the data. Nonetheless, these authors found a wide thermal breadth for aerobic metabolic performance, especially for acutely exposed killifish, and report a mismatch between  $T_{\text{opt,AS}}$  and specific growth rate for 'northern' killifish where growth was lowest at temperatures where AS was highest (25–30°C). These data support the ideas presented above for barramundi.

The idea that compensatory adjustments in AS occur to meet the challenges of daily life adds a possible, but yet to be confirmed, dimension to interpretations of performance within the OCLTT framework. As a eurythermal species, barramundi can experience widely fluctuating temperatures on a daily and seasonal basis which would select against a specific  $T_{\text{opt,AS}}$  and a fixed level of AS. Whether or not this means that barramundi will be resilient to climate warming remains to be seen, yet it does mean that the OCLTT concept and associated assumptions about cardiovascular limitations have limited power to predict climate change influences on barramundi and probably other eurythermal species. Based on these findings, and on the above-mentioned studies that also report exceptional aerobic performance of other animals at extreme high temperatures, it is suggested that the OCLTT hypothesis should be tested more rigorously before assumptions are made of its general relevance to water-breathing organisms. Large-scale studies that

combine measurements of AS with parameters such as growth and reproductive performance on an intraspecific level will help to tease apart correlation from causation in the context of OCLTT.

## MATERIALS AND METHODS

### Animals and holding conditions

In early February 2012, juvenile barramundi (*Lates calcarifer*) were obtained from Good Fortune Bay Hatchery, Bowen, Queensland, Australia. The pedigree of the fish was not known due to hatchery cross-breeding practices to maintain genetic diversity, yet it is most likely that the juveniles used in this study were the offspring of wild caught females fertilised by male broodstock that had been reared in captivity for one to three generations. The condition index ( $k=100,000M_b/L^3$ , where  $M_b$  is body mass in grams and  $L$  is total length in millimetres) of the fish used in this study ( $k=1.2$ – $1.5$ ) compared favourably with previously published values for wild caught individuals (Pusey et al., 2004) (and references within), indicating negligible influence of hatchery practices on the gross morphology of the fish. Due to the protandrous hermaphroditic nature of barramundi (Moore, 1979), all juveniles used in the present study were assumed to be males.

The fish were transported by road to the Australian Institute of Marine Science (AIMS), Townsville, Queensland, Australia, where they were held in 40 l rectangular tanks containing 30 l of seawater. The tanks were supplied with recirculated, filtered seawater (particle filtered and UV sterilised) at 29°C with a salinity of 35 ppt from a 250 l sump. Water in the sump was replaced with fresh seawater at a rate of 40 l h<sup>-1</sup> to minimise any build-up of waste products. Photoperiod was set to 13 h:11 h light:dark. Fish were fed to satiation every second day with Ridley Aqua-Feed Marine Float pellets (Ridley Corporation, Melbourne, Victoria, Australia).

The experiments carried out in this study were conducted in compliance with the James Cook University Animal Ethics Committee.

### Respirometry set-up

Rates of oxygen consumption ( $\dot{M}_{O_2}$ ) by the fish were obtained using automated intermittent-closed respirometry similar to that described previously (Clark et al., 2011; Norin and Malte, 2011) and using best practices outlined in Steffensen (Steffensen, 1989) and Clark et al. (Clark et al., 2013).

The experimental set-up consisted of polypropylene respirometers submerged in a darkened tank (experimental tank) containing 260 l of fully aerated seawater with a salinity of 35 ppt. Temperature was controlled by a water bath with a thermostat connected to a stainless-steel coil submerged in the experimental tank. Water in the experimental tank was continuously replaced with fresh seawater at a rate of 42 l h<sup>-1</sup>. Water was continuously circulated through each respirometer using an in-line Eheim pump in a recirculation loop, and the oxygen concentration of the respirometer water was measured continuously at 1 Hz in the recirculation loop using fibre optic leads focused on contactless oxygen sensor spots (Firesting O<sub>2</sub>, Pyro Science, Aachen, Germany). Automated flush pumps refreshed the water in the respirometers for 4–6 min in every 10 min period (flush cycle adjusted based on fish size and water temperature to ensure oxygen levels in the respirometers always remained above 80% air saturation) and  $\dot{M}_{O_2}$  was calculated from the decline in oxygen concentration in the respirometers between flush cycles.

### Respirometry protocol – acutely exposed fish

In mid-March 2012, seven barramundi (fasted for 44–48 h;  $M_b=25.4\pm1.9$  g; length= $125\pm4$  mm) were transferred from the holding tank (29°C) into seven of eight respirometers (1.43 l each) at 29°C using a water-filled plastic container to minimise stress associated with air exposure. Background respiration was quantified in each respirometer prior to introduction of the fish.  $\dot{M}_{O_2}$  measurements were begun immediately and continued until the following morning (~16 h), resulting in ~100  $\dot{M}_{O_2}$  measurements per fish from which the standard metabolic rate (SMR) was calculated (see below). The eighth respirometer was left without a fish and thus continually recorded background respiration.

The fish were then individually removed from the respirometers and placed in a circular tub containing 25 l of fully aerated seawater at 29°C.

MMR was induced in each fish using a standard protocol of 3 min manual chasing by the experimenter followed by 1 min of air exposure in a soft-meshed hand net (Clark et al., 2012). No individual could sustain burst swimming for greater than 2 min, and all individuals were physically exhausted following the protocol. Each fish was immediately placed back into the respirometer following the air exposure period and  $\dot{M}_{O_2}$  measurements were begun within 8 s and continued for at least 1.5 h. This protocol was selected for two main reasons. First, barramundi are ambush predators that do not undertake any sustained high-speed swimming at any point throughout their life cycle, such that speed increment tests in a swimming respirometer (i.e. a  $U_{crit}$  protocol) were inappropriate and unlikely to elicit MMR. Second, this approach is supported by previous studies on Atlantic cod, a species with a similar morphology and locomotor behaviour as barramundi, where a chase protocol induced a higher  $\dot{M}_{O_2}$  than a standard swim test in a tunnel respirometer (Reidy et al., 1995).

Upon completion of SMR and MMR measurements at 29°C, the experimental tank containing the respirometers was decreased to 23°C at a rate of 2°C h<sup>-1</sup> and then  $\dot{M}_{O_2}$  measurements were taken overnight (~16 h) to establish SMR at the new test temperature (~100  $\dot{M}_{O_2}$  measurements per fish). The rate of temperature change of 2°C h<sup>-1</sup> was chosen based on field measurements in barramundi habitats of tropical Australia (Loong et al., 2005). A MMR trial was repeated at the new test temperature the following morning using the same protocol as detailed above. The same SMR and MMR procedures were again repeated at 35°C (temperature again changed 2°C h<sup>-1</sup>), and then the temperature of the experimental tank was decreased to 29°C for a final assessment of SMR and MMR in comparison with the initial values obtained at 29°C (see Fig. 2 for an example of some of the respirometry protocol).

The fish were then returned to their holding tank and background respiration in each respirometer was again quantified. The fish were left to recover in their holding tank for 2 days after which they were fed to satiation. Once post-absorptive (~48 h post-feeding; 4 days after initial trials), the same respirometry protocol was repeated at 38°C (temperature increased from 29°C at 2°C h<sup>-1</sup>) to establish SMR and MMR at a temperature approaching the assumed upper thermal limit of the species (see Katersky and Carter, 2005; Newton et al., 2010). An additional fish ( $M_b=19.9$  g) was included in the eighth respirometer for these subsequent experiments, as the initial measurements with an empty respirometer revealed only minor background respiration. Nevertheless, assessments of background respiration were conducted in all respirometers and in all trials, both before and after the fish  $\dot{M}_{O_2}$  measurement protocol. Having performed well at 38°C, a final test temperature of 41°C was attempted. However,  $\dot{M}_{O_2}$  measurements lasted for only 2.5 h once 41°C was reached (15  $\dot{M}_{O_2}$  measurements per fish), after which some fish began losing equilibrium and the temperature of the experimental tank was rapidly decreased to 29°C for removal of all the fish. As the  $\dot{M}_{O_2}$  values at 41°C did not represent either SMR or MMR, they were not included in the data analysis but are shown in Fig. 1A for comparative purposes.

### Temperature preference experiment

To complement the  $\dot{M}_{O_2}$  data obtained at different temperatures, eight barramundi from the same cohort (acclimated to 29°C; length=187±3 mm) were individually tested for temperature preference in a figure-of-8-shaped acrylic shuttle tank (custom built at the AIMS) where two circular compartments (height 450 mm, diameter 500 mm, water depth 150 mm) were connected by a small passage (50 mm entrance, 80 mm length). Fully aerated water was supplied to each compartment from two external reservoirs, with the water in one compartment flowing in a clockwise and the other in a counter-clockwise direction (both 40 mm s<sup>-1</sup>). An overflow in each compartment led water back to the reservoirs and ensured a constant water level. The shuttle tank had darkened sides and black material was draped over the top during experiments to avoid any external stimuli. The reservoirs and shuttle tank were kept at 29°C while the fish was introduced into a compartment (alternating between trials) and for 1–2 h afterwards, then one compartment was heated to 38°C at a rate of ~6°C h<sup>-1</sup> via one external reservoir and the other compartment was maintained at 29°C with the assistance of a refrigeration unit supplying the other external reservoir. The circulating current inside each of the compartments in the shuttle tank

prevented mixing of the two water masses but a small inflow of hot water to the cold side, and vice versa, meant that a gradient of intermediate temperatures was available near the passage joining the compartments. This set-up allowed the fish to move freely between the two circular compartments with distinctly different water temperatures at the extremes (29 and 38°C). The low temperature (29°C) was chosen to match the acclimation temperature of the fish, while the high temperature (38°C) was chosen on the basis that it matched the temperature at which AS was found to be the largest.

The temperature of the fish in the shuttle tank was recorded every 2 min by a temperature logger (iButton, DS1921H, 0.125°C resolution; Maxim Integrated Products, Inc., San Jose, CA, USA) attached to the fish immediately below the dorsal fin with a single suture. To counteract the excess load imposed on the fish and provide a soft barrier between the logger and the fish's scales, a thin piece of high-density foam was attached to the base of the logger to make it neutrally buoyant before suturing it to the fish. Attachment of the logger occurred under clove oil anaesthesia (40 mg l<sup>-1</sup>) and the fish was left to recover in its holding tank for 3 days before the temperature preference experiment was commenced. All fish fed well during the recovery period, and a feed to half-satiation was given about 4 h before entry into the shuttle tank. Fish were given 12 h to settle in the shuttle tank after the water temperatures in each compartment had stabilised (~4 h after entry), after which all logger data were included in analyses (~56 h of data per fish). An experimenter visually checked each fish two to three times per day (ensuring not to disturb the fish) and on no occasion was a fish seen to be holding within the small passage connecting the two compartments, instead choosing to hold in the circular flow of one of the compartments. Each fish remained in the shuttle tank for a total of ~3 days before the logger was removed and the fish was returned to the holding tank.

### Respirometry protocol – acclimated fish

In November 2012, eight new fish from the same cohort ( $M_b=75.1\pm5.0$  g; length=189±4 mm) were acclimated to 38°C for 5 weeks (temperature changed from 29°C at ~3°C day<sup>-1</sup>) to test the effects of longer-term thermal acclimation on  $\dot{M}_{O_2}$ . An additional four fish ( $M_b=89.9\pm10.2$  g; length=199±8 mm) were maintained at their normal acclimation temperature of 29°C for the same period of time to monitor for any influence of longer-term captivity on  $\dot{M}_{O_2}$ . Fish were fed to satiation every second day, as above, and there were no discernible differences in the appetite or food consumption of individuals at the different temperatures. There were no mortalities during the acclimation period, and visually all fish remained in excellent health. Following acclimation and 48 h of fasting, fish were tested for SMR and MMR at their respective acclimation temperature (29 or 38°C) using the same respirometry protocol described above, except that the volume of each respirometer was 4.24 l (to account for the fact that the fish had grown) and only four respirometers were used per trial. Again, assessments of background respiration were conducted in all respirometers both before and after the fish  $\dot{M}_{O_2}$  trials. No attempts were made to test the fish at temperatures higher than 38°C.

### Data analysis and statistics

All  $\dot{M}_{O_2}$  data were analysed after importing the text file from the Firesting O<sub>2</sub> software into LabChart 7 (ADInstruments Pty Ltd, Bella Vista, New South Wales, Australia). Linear regressions between oxygen concentration and time were made for each measurement period and slopes derived from the regressions were used to calculate  $\dot{M}_{O_2}$  (mg min<sup>-1</sup> kg<sup>-1</sup>) after accounting for the volume of the respirometer and the volume and  $M_b$  of the fish. Although background respiration was minor, it was always accounted for (subtracted from  $\dot{M}_{O_2}$ ) when calculating  $\dot{M}_{O_2}$  by using actual values from the empty respirometer or, where all respirometers contained fish, by assuming a linear increase in background respiration between measurements taken at the beginning and end of an experiment.

SMR was determined by first taking the mean of the lowest 10% of  $\dot{M}_{O_2}$  measurements over the ~16 h period at each test temperature after which outliers (±2 s.d. from the mean) were excluded (no more than two data points were identified as outliers for any fish) and SMR was calculated as the mean of the remaining measurements. Note that  $\dot{M}_{O_2}$  data from the 41°C



trial are presented in Fig. 1A as all  $\dot{M}_{O_2}$  measurements for all fish (small grey circles) as well as the overall mean based on the lowest three measurements from each fish once 41°C was reached and before the fish lost equilibrium (larger grey circle with s.e.m. error bars). MMR was determined as the highest  $\dot{M}_{O_2}$  measurement recorded in any 2 min period at each test temperature (excluding 41°C), which always occurred immediately after the exhaustive exercise protocol.  $\dot{M}_{O_2}$  values were corrected for minor deviations in temperature (typically <0.05°C and never deviating more than 0.52°C from the experimental temperatures indicated above) using the calculated  $Q_{10}$  coefficient for either SMR or MMR (see Results). Regressions of residual SMR or MMR against  $M_b$  (range 28.5–107.8 g) did not yield significant relationships (data not shown), indicating that there was no allometric influence of  $M_b$  on the  $\dot{M}_{O_2}$  values reported herein. AS was calculated as MMR – SMR, and FAS was calculated as MMR/SMR.

Thermal preference for each fish was evaluated for ~56 h of logger data once the settling period had concluded (12 h after the temperature in the shuttle compartments had stabilised). The raw logger data from each fish was used to produce histograms and calculate median, mean and modal temperatures.

Statistical analyses were performed in SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA). Differences in SMR, MMR, AS and FAS between test temperatures for acutely exposed fish were analysed using one-way repeated measures ANOVA followed by a Holm–Šidák multiple comparison procedure. Comparisons between acclimated fish and acutely exposed fish were performed with *t*-tests. The overall level of significance was  $P < 0.05$  and *P*-values for pairwise comparisons of acutely exposed fish were adjusted according to the Holm–Šidák method. Values are presented as means ± s.e.m. unless otherwise indicated.

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#### Competing interests

The authors declare no competing financial interests.

#### Author contributions

T.N. and T.D.C. designed and conducted the experiments. T.N., H.M. and T.D.C. analysed and interpreted the data. T.N. and T.D.C. wrote the article.

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