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RESEARCH ARTICLE

Physiological responses of ectotherms to daily temperature variation

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ABSTRACT

Daily thermal fluctuations (DTFs) impact the capacity of ectotherms to maintain performance and energetic demands because of thermodynamic effects on physiological processes. Mechanisms that reduce the thermal sensitivity of physiological traits may buffer ectotherms from the consequences of DTFs. Species that experience varying degrees of DTFs in their environments may differ in their responses to thermally variable conditions, if thermal performance curves reflect environmental conditions. We tested the hypothesis that in response to DTFs, tadpoles from habitats characterised by small DTFs would show greater plasticity in the thermal sensitivity of physiological processes than tadpoles from environments characterised by large DTFs. We tested the thermal sensitivity of physiological traits in tadpoles of three species that differ naturally in their exposure to DTFs, raised in control (24°C) and DTF treatments (20–30°C and 18–38°C). DTFs reduced growth in all species. Development of tadpoles experiencing DTFs was increased for tadpoles from highly thermally variable habitats (~15%), and slower in tadpoles from less thermally variable habitats (~30%). In general, tadpoles were unable to alter the thermal sensitivity of physiological processes, although DTFs induced plasticity in metabolic enzyme activity in all species, although to a greater extent in species from less thermally variable environments. DTFs increased upper thermal limits in all species (between 0.89 and 1.6°C). Our results suggest that the impact of increased thermal variability may favour some species while others are negatively impacted. Species that cannot compensate for increased variability by buffering growth and development will probably be most affected.

KEY WORDS: Burst swimming performance, Critical thermal maximum, Daily thermal fluctuations, Enzyme activity, Metabolic rate, Tadpole

INTRODUCTION

How organisms respond to environmental temperature change will determine species persistence in variable environments. Temperature is well documented as the most pervasive abiotic factor to influence physiological function because of thermodynamic effects on biochemical reactions which underlie growth, reproduction and performance (Hochachka and Somero, 2002). For most ectotherms, environmental temperature determines body temperature (Guderley, 2004; Seebacher and Murray, 2007). Consequently, changes in ambient temperature affect physiology, altering individual performance and fitness. The ability of ectotherms to flexibly alter physiological mechanisms in response to changes in environmental temperature (plasticity/acclimation/acclimatisation) therefore determines their capacity to buffer performance and fitness from environmental variation (Seebacher et al., 2015).

Daily thermal fluctuations (DTFs) are particularly challenging for ectotherms. Because of the non-linear relationship between temperature and physiological processes, metabolic demands for cell maintenance are increased at high temperatures (Ruel and Ayres, 1999). As such, DTFs increase metabolic demands compared with constant temperature conditions, causing energy trade-offs that can affect growth and development (Niehaus et al., 2012; Arrighi et al., 2013; Colinet et al., 2015). Furthermore, as temperature changes during the day, animals may be unable to maintain important physiological and performance traits such as growth, foraging and predator avoidance. During development, DTFs can increase energetic demands, which results in decreased rates of development (Niehaus et al., 2006; Dhillon and Fox, 2007; Les et al., 2007), and reduced body size at maturity compared with animals developing at the equivalent mean temperature (Atkinson, 1996; Dong et al., 2006; Niehaus et al., 2006; Dhillon and Fox, 2007). In some species, however, DTFs can increase body size and rate of development (Dong et al., 2006; Du and Feng, 2008; Folguera et al., 2011) as responses to DTFs are highly variable between species/traits.

Ectotherms exposed to DTFs would benefit from reducing the thermal sensitivity of metabolism in order to buffer energetic demands associated with fluctuating temperatures (Huey and Hertz, 1984; Gabriel et al., 2005). Reversible plasticity should develop in heterogeneous environments when the stressor occurs somewhat regularly, associated with a reliable environmental cue (Relyea, 2002; Gabriel et al., 2005). Under which conditions DTFs provide a reliable cue for inducing plasticity in the thermal sensitivity of traits, and when this will be beneficial has not been established (Sinclair et al., 2006; Niehaus et al., 2011; Williams et al., 2012). Individuals can reduce the thermal sensitivity of metabolism and performance in response to DTFs (Dame and Vernberg, 1978; Měráková and Gvoždík, 2009; Williams et al., 2012). For example, butterfly larvae of *Erynnis propertius* appear to have the capacity to change the thermal sensitivity of metabolism as an acclimation response to the degree of DTFs experienced in different microclimates (Williams et al., 2012). In response to DTFs, individuals can also increase thermal tolerance, reducing damage caused by temperature extremes (Feldmeth et al., 1974; Schaefer and Ryan, 2006). However, some species show no change in thermal sensitivity in response to DTFs (Niehaus et al., 2011; Kern et al., 2014).

As with other forms of plasticity, what drives the response to DTFs may be the degree of thermal variability an organism experiences in its environment (Relyea, 2002; Gabriel et al., 2005). Ectotherms from environments with little diurnal temperature variation would benefit from mechanisms that reduce the thermal sensitivity of physiological traits in response to increased DTFs, in

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order to reduce the metabolic cost of exposure to high temperatures (Sinclair et al., 2006; Williams et al., 2012). Ectotherms from environments characterised by large DTFs may already have thermally insensitive physiological rates as a result of selection pressures of a highly variable environment (Huey and Hertz, 1984). As such, low thermal sensitivity of physiological rate processes across the range of environmental temperatures experienced may limit the benefits of plasticity in response to DTFs (Williams et al., 2012). When DTFs extend beyond the normal range of temperature fluctuations experienced, animals with the capacity to reduce their thermal sensitivity may be more robust than those with non-plastic phenotypes. Determining what shapes physiological responses to DTFs may identify species that are capable of overcoming energetic consequences of short-term temperature variation. Animals that show plasticity in response to high rates of temperature change may be less affected by environmental perturbation.

Anurans provide a good model to investigate responses to DTFs, as the tadpoles of related species can develop in water bodies characterised by widely different thermal conditions, i.e. dams, lakes, streams and ephemeral pools. We studied the tadpoles of three related species of Australian frogs (*Limnodynastes peronii*, *Limnodynastes tasmaniensis* and *Platyplectrum ornatum*), whose developmental environments vary in the degree of DTF as a result of habitat type and distribution. We hypothesised that the capacity to reduce the thermal sensitivity of physiological and performance traits would be associated with the degree of daily thermal variability in the habitats of different species. Specifically, we predicted that tadpoles that experience less daily thermal variability in their environment would exhibit a greater capacity to reduce the thermal sensitivity of physiological processes in response to DTFs than tadpoles from highly thermally variable environments. This comparison may allow us to understand whether environmental variability determines physiological responses to DTFs.

RESULTS

Water temperature

The mean water temperature of shallow pools at *P. ornatum* collection sites was 24.7±0.3°C, with average daily fluctuations of 17.5±0.3°C. Deep pools at the geographical location where *L. tasmaniensis* eggs were collected had a mean temperature of 23.9±0.2°C and average daily fluctuations of 13.2±0.1°C. Water temperature at *L. peronii* collection sites had a mean of 27.3±0.1°C and average daily fluctuations of 5.9±0.4°C (Fig. 1).

Mortality, development time and body condition

No tadpoles of *L. peronii* or *L. tasmaniensis* in the large DTF treatment survived. Mortality of *L. tasmaniensis* tadpoles in the small DTF treatment was significantly higher (58%) than that of tadpoles in the control treatment (39%, N=340, χ^2_2=12.79, P<0.001). Mortality of *L. peronii* tadpoles was not different between the small DTF treatment (38%) and the control treatment (29%). Mortality of *P. ornatum* tadpoles was not different between temperature treatments (control 53%, small DTF 51%, large DTF 49%).

The time taken to reach development stage 35–37 was longer for tadpoles in the small DTF treatment compared with those in the control treatment for *Limnodynastes* species (*L. peronii*, N=192, χ^2_1=135.11, P<0.001; *L. tasmaniensis*, N=163, χ^2_1=118.97, P<0.001). Conversely, development time was shorter for *P. ornatum* tadpoles in both fluctuating treatments compared with the control (N=245, χ^2_2=32.86, P<0.001). For *P. ornatum* tadpoles, the time to reach development stage 35–37 was not different between the small and large DTF treatments (Table 1).

Body mass (M_b) and body length (BL) of *L. peronii* tadpoles was not significantly affected by treatment, whereas tail length (TL) was significantly reduced by small DTFs (Table 1; TL, $F_{1,188}$=8.27, P=0.005). For both *L. tasmaniensis* and *P. ornatum*, M_b, BL and TL

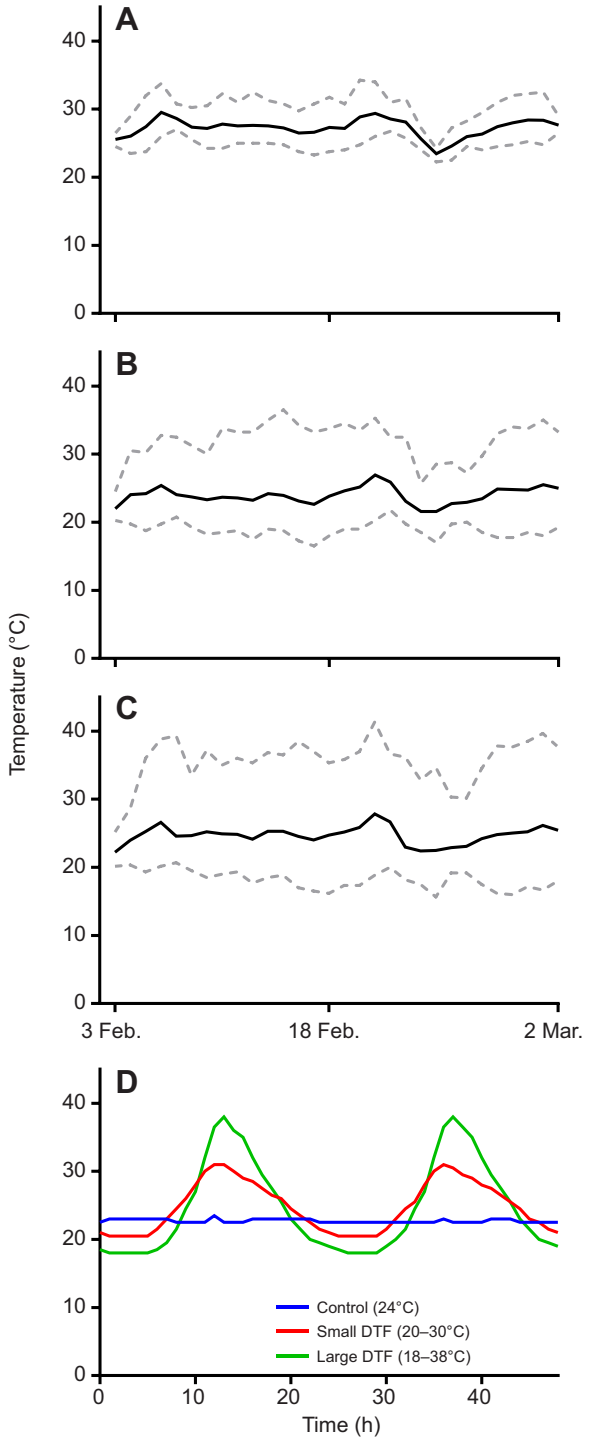

Fig. 1. Habitat temperatures of the study species and treatment temperatures. Water temperature at a depth of ~10 cm was recorded every half hour for 1 month at collection sites of (A) *Limnodynastes peronii*, (B) *Limnodynastes tasmaniensis* and (C) *Platyplectrum ornatum*. Black lines represent daily mean temperatures, while dashed lines represent the daily maximum and minimum temperatures. (D) Treatment temperatures reflect habitat temperatures; control treatment (24°C), and small daily thermal fluctuation (DTF; 20–30°C) and large DTF (18–38°C) treatments.

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Table 1. Morphometrics and time taken to reach development stage 35–37 of tadpoles raised in control, small DTF and large DTF treatments

	Treatment (°C)	Development* (days)	M_b (g)	BL (mm)	TL (mm)
Limnodynastes peronii	24	90.79±0.72^{q}	338.24±7.41	12.44±0.1	26.20±0.3^{c}
	20–30	115.55±0.86^{b}	320.95±5.4	12.46±0.1	25.12±0.25^{d}
Limnodynastes tasmaniensis	24	156.43±1.1^{e}	463.13±8.92^{g}	14.16±0.12^{i}	28.03±0.24^{k}
	20–30	200.93±0.65^{f}	370.12±9.24^{h}	13.46±0.13^{j}	24.10±0.25^{l}
Platyplectrum ornatum	24	29.75±0.38^{m}	243.26±6.16^{o}	11.48±0.12^{r}	19.77±0.21^{u}
	20–30	25.60±0.33^{n}	182.66±4.62^{p}	10.46±0.11^{s}	17.78±0.22^{v}
	18–38	25.74±0.46^{n}	140.45±3.6^{q}	9.67±0.09^{t}	17.02±0.2^{w}

DTF, daily thermal fluctuation; M_b, body mass; BL, body length; TL, tail length.
For treatment: 24°C, control; 20–30°C, small DTFs; 18–38°C, large DTFs.
*Time taken to reach development stage 35–37.
Different letters denote significant differences.

were all significantly reduced in tadpoles in small DTFs (Table 1; *L. tasmaniensis* log M_b, $F_{1,161}$=54.93, P<0.001; BL, $F_{1,161}$=17.66, P<0.001; TL, $F_{1,161}$=121.57, P<0.001; *P. ornatum* sqrt M_b, $F_{2,160}$=109.97, P<0.001; BL, $F_{2,241}$=75.44, P<0.001; TL, $F_{2,241}$=54.9, P<0.001) and all morphological traits were further reduced in *P. ornatum* tadpoles raised in large DTFs (Tukey HSD, all comparisons P<0.05).

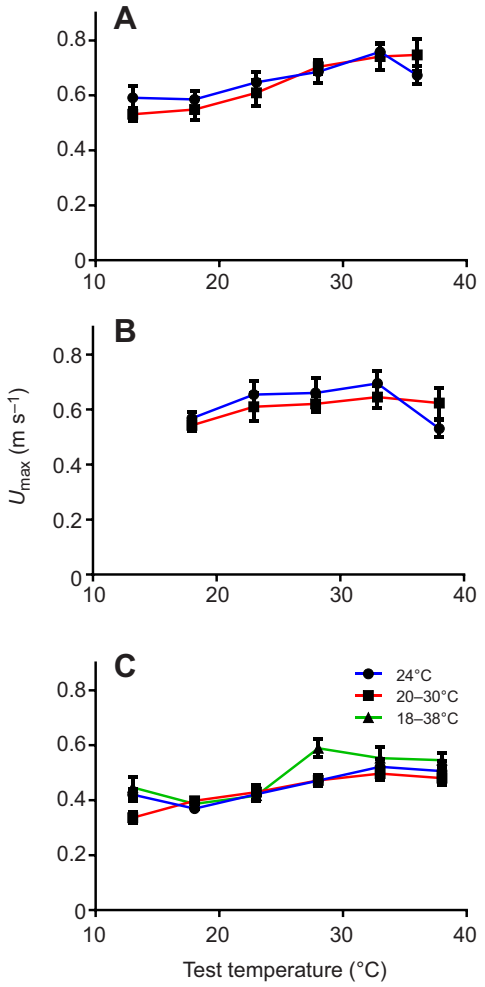

Fig. 2. Maximum burst swimming performance of tadpoles raised in control and fluctuating temperatures. Maximum burst swimming performance (U_{max}, means±s.e.m.) of tadpoles was tested at 13, 18, 23, 28, 33 and 36/38°C. Swimming performance of (A) *L. peronii*, (B) *L. tasmaniensis* and (C) *P. ornatum* tadpoles was not different between control (24°C), small DTF (20–30°C) and large DTF (18–38°C) treatments.

Maximum burst swimming performance

Maximum burst swimming performance (U_{max}) was not significantly affected by treatment in any species (Fig. 2). Test temperature significantly affected swimming performance for *L. peronii* ($F_{1,89}$=13.37, P=0.04) and *L. tasmaniensis* ($F_{1,75}$=6.37, P=0.01) through the *y*-intercept parameter.

Resting metabolic rate

The small DTF treatment significantly affected the thermal performance curve (TPC) for resting metabolic rate (RMR) of *L. tasmaniensis* through the shape of the apex (*a*), the slope (*b*) and the *y*-intercept (*c*; $y=ax^2+bx+c$) (Table 2). This resulted in reduced thermal sensitivity at low test temperatures, and increased metabolic rate at the highest test temperature compared with tadpoles from the control temperature treatment (Fig. 3). There was no effect of treatment on RMR of the other species. Test temperature affected RMR through the *y*-intercept for *L. tasmaniensis* (Fig. 3) and the slope of RMR for *L. peronii* ($F_{1,87}$=6.95, P=0.01), but RMR was not affected by test temperature in *P. ornatum*.

Metabolic enzyme activity

In *L. peronii* tadpoles, the activity of lactate dehydrogenase (LDH) and citrate synthase (CS) enzymes was significantly higher in tadpoles from the small DTF treatment than in tadpoles from the control treatment (Fig. 4A,B; supplementary material Fig. S1). The activity of LDH in tadpoles from the small DTF treatment showed significant curvature in response to test temperature ($F_{1,111}$=10.33, P<0.001). The activity of CS increased with test temperature ($F_{1,109}$=1744.04, P<0.001), and was significantly higher in tadpoles from the small DTF treatment compared with tadpoles from the control treatment ($F_{1,21}$=6.07, P=0.02). The activity of cytochrome *c* oxidase (COX) was significantly affected by test temperature ($F_{1,118}$=401.75, P<0.001), but was not significantly different between treatments (Fig. 4C).

In *L. tasmaniensis* tadpoles, the activity of all metabolic enzymes was higher in tadpoles from the small DTF treatment compared with that in tadpoles from the control treatment (Fig. 4D–F). The activity of CS increased with test temperature ($F_{1,119}$=1788.77, P<0.001), and was significantly higher in tadpoles from the small DTF treatment compared with that in tadpoles from the control treatment ($F_{1,22}$=6.72, P=0.02; Fig. 4E). Between treatments, the activity of LDH and COX was affected by an interaction between treatment and the polynomial term (LDH, $F_{2,116}$=72.77, P<0.001; COX, $F_{2,116}$=7.45, P<0.001) inferring different rate curves in response to temperature change. LDH activity was significantly higher at high test temperatures in tadpoles in the small DTF treatment, while COX activity was significantly higher in tadpoles from the small DTF treatment across the range of test temperatures (supplementary material Fig. S1).

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Table 2. ANOVA from quadratic regression on RMR data

	a			*b*			*c*		
	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value
Test temperature	1,74	0.93	0.34	1,74	2.99	0.09	1,74	26.75	<0.0001
Treatment	1,74	4.85	0.03	1,74	5.07	0.03	1,74	5.84	0.02

Data are for parameters from the function: $y=ax^2+bx+c$.
There was a significant effect of treatment and test temperature on resting metabolic rate (RMR) in *L. tasmaniensis*.

In *P. ornatum* tadpoles, only the activity of CS was significantly different between treatments (Fig. 4H). CS activity was significantly lower in tadpoles from the large DTF treatment compared with that in tadpoles from the control and small DTF treatment, and CS activity in the small DTF treatment was significantly lower than control between test temperatures of 18 and 33°C. The activity of CS and COX was affected by an interaction between treatment and the polynomial term (CS, $F_{4,154}$=97.83, P<0.001; COX, $F_{4,154}$=6.20, P<0.001). There was no effect of treatment on COX activity (Fig. 4I; supplementary material Fig. S1). In tadpoles from the control and large DTF treatments, the activity of LDH increased linearly with increasing test temperature, while the LDH activity of tadpoles in the small DTF treatment increased with significant curvature in response to increasing test temperature ($F_{2,154}$=495.88, P<0.001), but there was no significant difference between treatments.

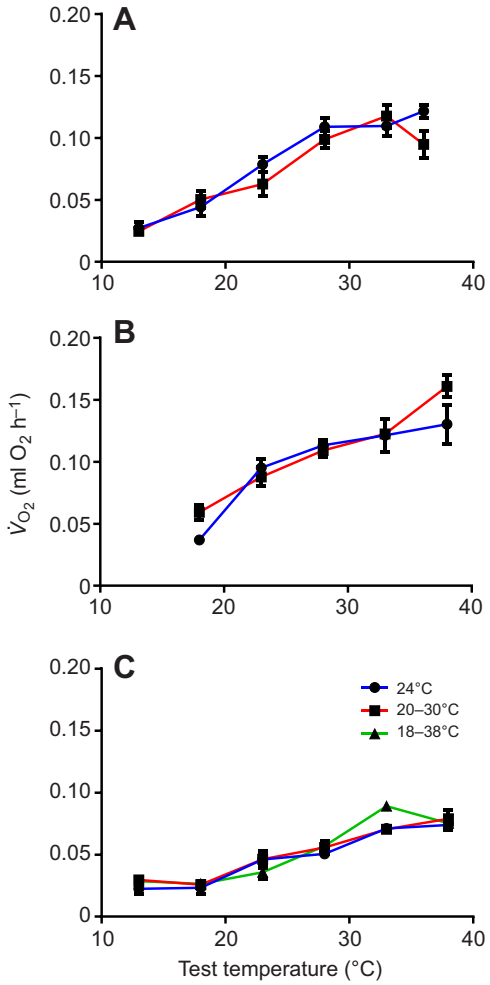

Fig. 3. Oxygen consumption of tadpoles raised in control and fluctuating temperatures. Rate of oxygen consumption ($\dot{V}_{O_2}$; means±s.e.m.) of tadpoles was tested at 13, 18, 23, 28, 33 and 36/38°C. $\dot{V}_{O_2}$ was not different between tadpoles of (A) *L. peronii* and (C) *P. ornatum* raised in stable or fluctuating treatments. Oxygen consumption of (B) *L. tasmaniensis* was significantly higher at low and high test temperatures for tadpoles raised in small DTF conditions (20–30°C) compared with tadpoles raised at control temperatures (24°C).

Critical thermal maximum

Tadpoles of all three species in the small DTF treatment had a significantly higher critical thermal maximum (CT_{max}) than tadpoles in the control treatment (Fig. 5; *L. peronii*, $F_{1,14}$=18.236, P<0.001; *L. tasmaniensis*, $F_{1,15}$=52.52, P<0.001; *P. ornatum*, $F_{2,16}$=37.80, P<0.001). CT_{max} for *P. ornatum* was also significantly higher in tadpoles in the large DTF treatment compared with those in control treatment (Tukey HSD P<0.001) but there was no difference between the CT_{max} of tadpoles that experienced small and large DTFs. *Platyplectrum ornatum* showed a significant interaction between treatment and M_b for this trait ($F_{1,16}$=4.25, P=0.03).

DISCUSSION

In response to diurnal temperature fluctuations, tadpoles showed plasticity in metabolic enzyme activity, but were unable to reduce the thermal sensitivity of physiological traits in order to buffer energetic demands. As a result, tadpoles experiencing DTFs had reduced growth compared with tadpoles in constant temperature in all species. Increased energy demands are an important consequence of short-term temperature variation because of the reduction in body size affecting the fitness of anurans by increasing vulnerability to predators (Wilson and Franklin, 2000; Wilson et al., 2000b; Kingsolver and Huey, 2008) and reducing fecundity at maturity (Sweeney and Schnack, 1977). DTFs also had consequences for development, although responses were different between species. The length of development was longer in *L. peronii* and *L. tasmaniensis* tadpoles exposed to small DTFs compared with those in constant temperature, further highlighting the energetic consequences of these thermal conditions. The opposite was true for *P. ornatum* tadpoles, which increased development in response to both small and large DTFs. The capacity to increase development may reflect plastic responses to their highly variable developmental environments.

Species such as *P. ornatum*, which develop in highly variable ephemeral pools, can increase the rate of development in response to cues that indicate drying of their habitat (Newman, 1989; Székely et al., 2010). Decreasing water level, crowding effects and low food availability have been shown to increase development at a cost to size at metamorphosis (Newman, 1989; Brady and Griffiths, 2000; Doughty and Roberts, 2003; Székely et al., 2010). This allows larvae to reach metamorphosis before their habitat desiccates. DTFs may also be a cue for increased rate of development, as thermal fluctuations would increase as water evaporates from ephemeral pools. Increased temperature variability may therefore reduce survival of species inhabiting temporal aquatic habitats if they are

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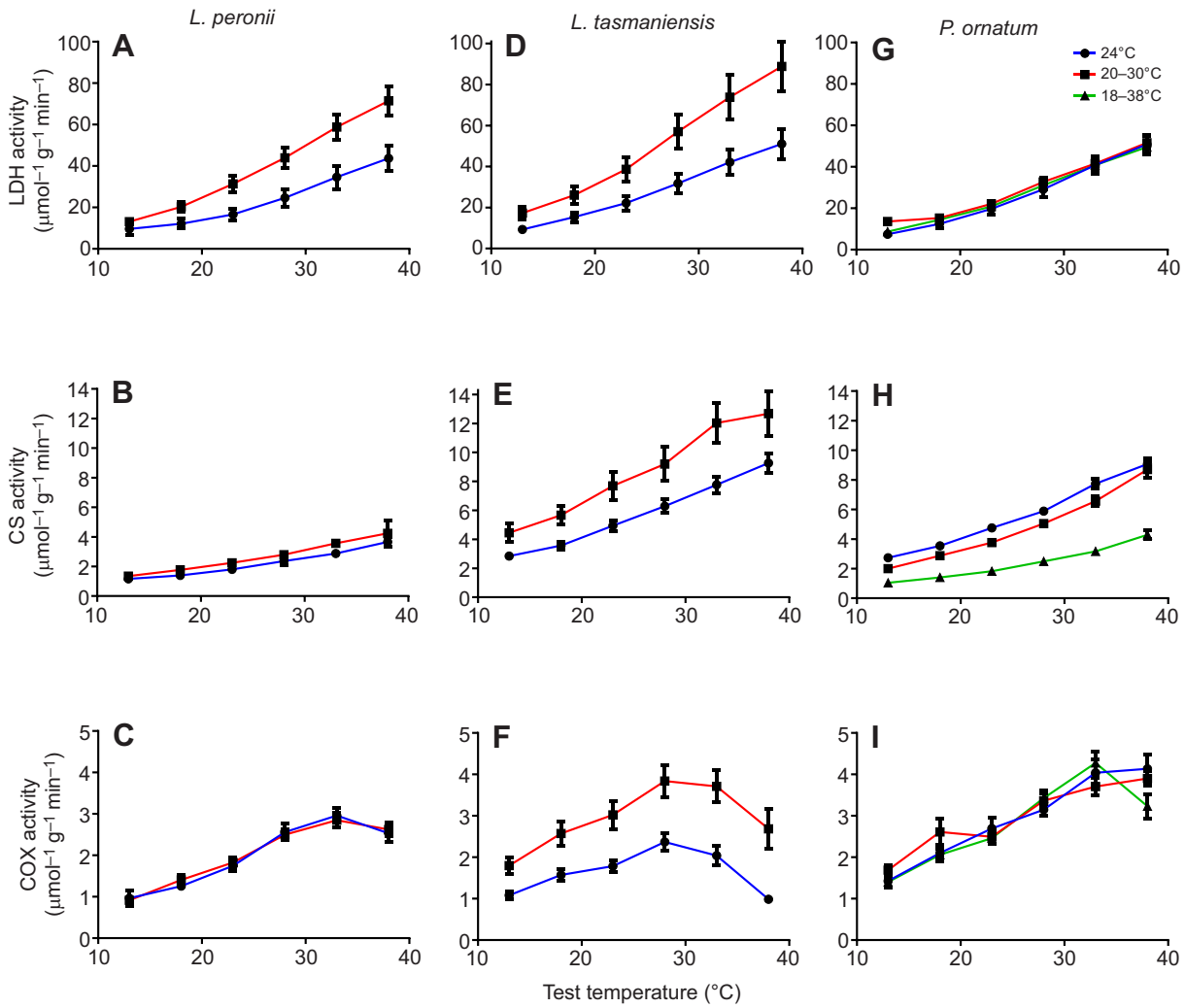

Fig. 4. Metabolic enzyme activity of tadpoles raised in control and fluctuating temperatures. Enzyme activity of lactate dehydrogenase (LDH), citrate synthase (CS) and cytochrome *c* oxidase (COX) for *L. peronii* (A–C), *L. tasmaniensis* (D–F) and *P. ornatum* (G–I) tadpoles raised in constant (24°C), small DTF (20–30°C) and large DTF (18–38°C) treatments, tested at 13, 18, 23, 28, 33 and 38°C.

unable to increase development in response to DTFs and metamorphose before habitats dry.

Different consequences of DTFs on development rate among species may be determined by differences in their thermal optimum for development (Bozinovic et al., 2011). When the mean temperature occurs close to a species' thermal optimum, temperature fluctuations force animals to spend time at temperatures below or above the thermal optimum, reducing the rate of development. However, if the mean of the thermal fluctuation is below the thermal optimum, the high temperatures experienced during temperature fluctuations will be closer to the thermal optimum and will increase the mean rate of development (Ruel and Ayres, 1999; Bozinovic et al., 2011; Colinet et al., 2015). Differences in the thermal optimum for development of the species studied may explain contrasting effects of DTFs on the length of development.

All species showed limited responses to DTFs in performance and metabolic rate. Across the species investigated there was no plasticity in burst swimming performance and only *L. tasmaniensis* tadpoles showed plasticity in RMR in response to DTFs. In response to small DTFs, *L. tasmaniensis* tadpoles reduced the thermal sensitivity of RMR at low temperatures, which may compensate for rate-limiting effects of cold temperatures. This maintenance of RMR at low

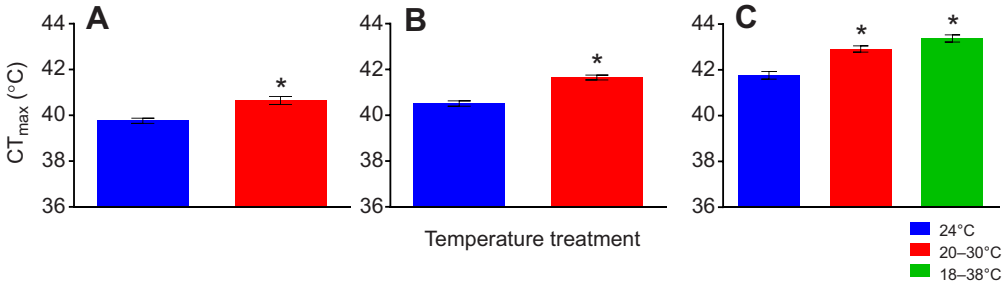

Fig. 5. Critical thermal maxima (CT_{max}) of tadpoles raised in stable and fluctuating temperatures. CT_{max} of tadpoles of (A) *L. peronii*, (B) *L. tasmaniensis* and (C) *P. ornatum* was significantly greater for tadpoles raised in small DTF (20–30°C) and large DTF (18–38°C) conditions compared with tadpoles in the control treatment (24°C). Asterisks denote significant differences between treatments.

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temperatures, however, was coupled with greater thermal sensitivity at high temperatures compared with tadpoles raised in stable conditions. As a result, there may have been increased costs associated with high temperatures and this may account for the increased mortality in response to small DTFs in this species. Reduced thermal sensitivity at high or low temperatures in response to DTFs has previously been demonstrated and may buffer some species from the most challenging environmental temperatures experienced in a thermally variable environment (Dame and Vernberg, 1978; Měráková and Gvoždík, 2009; Niehaus et al., 2011). Overall, however, the species investigated here were unable to buffer performance and metabolic rate from the effects of DTFs through plasticity in the shape of their TPCs.

DTFs did induce plasticity in metabolic enzyme activity, although this response also differed between species. Tadpoles of *P. ornatum* showed little change in enzyme activity overall, but had reduced CS activity after exposure to large DTFs. *Limnodynastes peronii* and *L. tasmaniensis* tadpoles increased aerobic metabolic enzyme activity (CS and COX, and CS, respectively) in response to small DTFs; however, this was not reflected by RMR. Therefore, changes in metabolic enzyme activity do not directly correlate to changes in metabolism. RMR indicates maintenance costs whereas the activity of COX and CS represents potential metabolic energy as they are rate-limiting enzymes in mitochondrial respiration (St-Pierre et al., 1998; Seebacher et al., 2014). The increase in enzyme activity in *Limnodynastes* species may provide a greater metabolic scope that would help meet metabolic demands for growth in a thermally variable environment. LDH activity is associated with anaerobic production of ATP (Guderley, 2004), so it is surprising that the increased activity of LDH in *L. peronii* and *L. tasmaniensis* tadpoles exposed to small DTFs did not correspond to changes in burst swimming performance. The lack of continuity between the responses of metabolic enzyme activity, RMR and swimming performance highlight the complexity of eliciting physiological responses, and that high order traits are probably dependent on complex interactions between cell- and tissue-level processes (Seebacher et al., 2010). Plasticity in metabolic enzyme activity alone does not buffer animals from the negative consequences of DTFs through plasticity in the shape of their TPCs.

Altered metabolic enzyme activity in tadpoles in response to DTFs shows that DTFs did provide a cue for plasticity in these traits. As *Limnodynastes* species increased enzyme activity rather than decreasing the thermal sensitivity of these traits as predicted, it is worth considering what aspect of DTFs acted as a cue for plasticity. The increase in enzyme activity in response to small DTFs reflects a cold acclimation response (Hofmann and Todgham, 2010; Seebacher et al., 2014). As tadpoles in thermally variable treatments spent more time below the mean temperature than above it, it is possible that the cue for acclimation was the cold overnight (modal) temperature, rather than the mean temperature in this case. This may also explain why *L. tasmaniensis* reduced thermal sensitivity of RMR at low temperatures, as cold acclimation can increase performance at low temperatures only (Wilson et al., 2000a). Our understanding of how animals respond to temperature variation would benefit from establishing what cues animals respond to in variable environments. Understanding what drives changes in enzyme kinetics and interactions between cellular processes to elicit plasticity would reveal more about how animals respond to variable environments and the mechanisms that elicit these responses.

DTFs induced plasticity in the upper thermal limits of all species. Tadpoles exposed to DTFs had higher upper thermal limits than those in constant temperature conditions. Plasticity of temperature tolerance in response to DTFs can buffer tadpoles from cellular damage, and lethal effects of peak environmental temperatures (Feldmeth et al., 1974; Otto, 1974; Schaefer and Ryan, 2006; Colinet et al., 2015).

What determines the capacity for thermal acclimation is complex, and may be dependent on several factors including phylogenetic history and environmental conditions at multiple time scales (Seebacher et al., 2012). *Limnodynastes peronii* and *L. tasmaniensis* are closely related yet experience a greater difference in environmental temperatures than that between *L. tasmaniensis* and *P. ornatum*. Phylogenetic relatedness may therefore explain similar changes in enzyme activities in response to DTFs. In theory, the capacity for plasticity is determined by the amount of environmental variation experienced relative to generation time (Angilletta, 2009). As *Limnodynastes* species have a long development time (months), they may experience mean temperature change within generations. These species are therefore expected to have the capacity for reversible plasticity in response to seasonal temperature change (Angilletta, 2009). The two *Limnodynastes* species may have perceived cues from stable elements from the diurnal temperature cycle to which they responded by altering enzyme activity, and RMR in the case of *L. tasmaniensis*. In contrast, the rapid development of *P. ornatum* may limit the amount of temperature variation experienced within generations and may potentially limit the benefits of thermal acclimation capacity. High DTFs in the environment could mask stable temperature cues and reduce environmentally induced plasticity, leading instead to selection of a broad performance curve (Huey and Hertz, 1984). This may be true for *P. ornatum*, which has thermally insensitive swimming performance and RMR (Kern et al., 2014) which may enable this species to maintain performance and buffer metabolic demands from DTFs inherent in their environment.

The consequences of increased temperature variability may be as important as mean temperature change. Acclimation allows animals to overcome (to varying degrees) the challenges associated with mean temperature change (Wilson and Franklin, 1999; Seebacher et al., 2014; Seebacher and Grigaltchik, 2014). Many ectotherms, however, appear to lack the capacity to physiologically respond to DTFs in a way that allows them to prevent increased metabolic demands associated with peak environmental temperatures (Henry and Houston, 1984; Kingsolver et al., 2009; Niehaus et al., 2011; Kjörsgaard et al., 2013; Kern et al., 2014). In this study, DTFs increased upper thermal limits, which may buffer tadpoles from the lethal consequences of temperature extremes. However, the inability to buffer metabolism from DTFs meant that growth and development (in *Limnodynastes* species) were negatively impacted. Importantly, different species exhibit different responses to DTFs and this is likely to influence the effects of climate change on ecological communities. Increased environmental variability associated with climate change (IPCC, 2013) may favour some species while others are negatively impacted. Species that cannot compensate for increased variability by buffering growth and development will probably be most affected. Understanding the responses of species to short-term temperature fluctuations may help to reveal how species respond to environmental change.

MATERIALS AND METHODS

Study species

We investigated the response to DTFs in tadpoles of three species that develop in different thermal environments; *Limnodynastes peronii* (Duméril and Bibron 1841), *L. tasmaniensis* (Günther 1858) and *Platyplectrum*

ornatum (Gray 1842). All three species are from the subfamily Limnodynastinae (Pyron and Wiens, 2011), which are characterised by building foam nests. *Platyplectrum ornatum* inhabit dry environments and breed after heavy rain in highly ephemeral water bodies characterised by large DTFs (>20°C; Anstis, 2002; Kern et al., 2014). The tadpoles of this species have low thermal sensitivity for burst swimming performance (Kern et al., 2014). *Limnodynastes peronii* and *L. tasmaniensis* are usually associated with permanent and semi-permanent water bodies that experience smaller DTFs (Fig. 1). These species can breed successfully in a range of habitats from permanent dams and lakes to ephemeral flooded grasslands and pools (Anstis, 2002). *Limnodynastes peronii* tadpoles have the capacity to acclimate to stable temperatures and have been shown to have thermally sensitive TPCs in the early stages of development (Wilson and Franklin, 1999; Niehaus et al., 2011; Seebacher and Grigaltchik, 2014).

Animal collection

Four partial egg masses of *L. tasmaniensis* and *P. ornatum* were collected from flooded road sides (in January and March, respectively) near Dalby, QLD, Australia (27°19′ S, 151°05′ E). Eggs of the former were found in deeper water bodies, while those of the latter were found in very shallow pools (personal observation). Three partial clutches of *L. peronii* eggs were collected from water bodies (in May) in St Lucia, QLD, Australia (27°30′ S, 152°59′ E). After collection, egg masses were transported to The University of Queensland. Water temperature at a depth of ∼10 cm was recorded at collection sites every hour for 1 month (iButton, Maxim Integrated Products Inc., San Jose, CA, USA).

Experimental treatments

Eggs were separated and placed individually into 80 ml containers in chemically aged water (Prime, Seachem, Madison, GA, USA), and randomly allocated between treatments. Tadpoles developed in one of three temperature treatments (each had a mean of 24°C); control (24°C), small DTFs (20–30°C) and large DTFs (18–38°C), based on observations of temperature variability recorded in habitats at collection sites (Fig. 1). Eggs were introduced to temperature treatments on the evening of collection when temperature cycles reached 24°C. Tadpoles were kept on a 14 h:10 h (light: dark) photoperiod and fed daily with boiled spinach, and containers were cleaned twice a week. Tadpoles developed in these conditions until they reached developmental stage 35–37 (Gosner, 1960). Stage 35–37 is a relatively stable time in development (Gosner, 1960) before hindlimbs are large enough to affect swimming movement (Hoff and Wassersug, 2000). At this developmental stage, tadpoles (N=7–9 per temperature/trait) were tested from each treatment either for CT_{max} or at one of six temperatures for RMR or U_{max}. We then recorded M_b (in g), BL and TL (in mm) and the number of days to reach development stage 35–37. After testing, tadpoles were killed by exposure to Aqui-S (175 mg l^{-1} Aqui-S, New Zealand Ltd). Tail muscle was dissected out and stored at −80°C for determination of metabolic enzyme activity. Mortality (%) was recorded daily through the experiment.

U_{max}

U_{max} was assessed at six temperatures (13, 18, 23, 28, 33 and 36 or 38°C; *L. peronii* did not survive in 38°C and so were tested at 36°C) in *L. peronii* and *P. ornatum* to generate a TPC. *Limnodynastes tasmaniensis* was tested at five temperatures (18, 23, 28, 33 and 38°C). Tadpoles were removed from temperature treatments when the temperature was ∼24°C. To prevent thermal shock, tadpoles were brought to the test temperature at a rate of 4°C h^{-1} and allowed to adjust to the test temperature for 1 h. Burst swimming performance was assessed in a swimming arena (27×13×5 cm) lined with reflective tape to give a clear silhouette of each tadpole. This container was filled with dechlorinated aged tapwater to a depth of 3 cm to prevent vertical movement, and semi-submerged in a waterbath set to the test temperature. Startle responses (C-start responses) were elicited by touching the tadpole's head with a blunt probe and were recorded using a high-speed digital camera (Canon EX-FH25, 240 Hz) pointed at a mirror positioned at a 45 deg angle above the burst arena. The first 200 ms (50 frames) following the completion of the C-start were analysed (Tracker Video Analysis and Modelling Tool, Open Source Physics; Alton et al., 2011) frame by frame, by digitising the snout to determine maximum velocity. Three startle responses were recorded for each tadpole and individual burst swimming data were smoothed using a generalised cross-validatory quantic spline filter (Walker, 1998). The fastest burst was recorded as U_{max}.

RMR

RMR was calculated from oxygen consumption using closed-system respirometry (Sinclair et al., 2006) at six test temperatures (13, 18, 23, 28, 33 and 36/38°C) to generate a TPC for *L. peronii* and *P. ornatum*. *Limnodynastes tasmaniensis* were tested at five temperatures (18, 23, 28, 33 and 38°C). As for swimming performance, tadpoles were removed from treatments and brought to each test temperature slowly to prevent thermal shock. Tadpoles were then placed individually into 25 ml plastic respirometers (syringes) filled with air-saturated, dechlorinated aged water. Respirometers were submerged in a waterbath set to the test temperature (±0.5°C), and after 10 min to allow tadpoles to recover from handling, respirometers were sealed with three-way taps and left for 40–60 min, depending on the test temperature (higher temperatures require less time). The respirometers were fitted with an oxygen-sensitive fluorescent Sensor Spot (PreSens, Regensburg, Germany) and aquatic oxygen partial pressure was determined non-invasively by measuring the fluorescence of the sensor spot through the plastic wall of the respirometer. A fibre-optic cable connected to a Fibox3 reader was used to capture and record fluorescence readings. Continuous, simultaneous temperature recordings of the waterbath allowed for the correction of O_2 solubility with changing water temperature.

The rate of oxygen consumption ($\dot{V}_{O_2}$: ml O_2 h^{-1}) was calculated using the following formula:

$$\dot{V}_{O_2} = (\Delta O_2 \times V)/T, \quad (1)$$

where ΔO_2 is the change in oxygen in the chamber (ml O_2 l^{-1}), V is the volume of the respirometer container (ml) and T is time (h).

Metabolic enzyme activity

We measured the activity of one enzyme involved in anaerobic metabolism, LDH, and two enzymes involved in aerobic respiration, COX and CS. We used tail muscle dissected from tadpoles for swimming performance and metabolic rate measurements (N=10–12). Tissue samples (0.022–0.053 g) were homogenised in lysis buffer (1:20 CS and COX, 1:100 LDH; 50 mmol l^{-1} imidazole, 2 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} EDTA, 0.1% Triton X-100 and 1 mmol l^{-1} glutathione, pH 7.5) and enzyme assays were conducted according to published protocols (Seebacher et al., 2003). Individual tadpoles were tested for activity of all three enzymes at 13, 18, 23, 28, 33 and 38°C.

CT_{max}

CT_{max}, the temperature at which animals lose the ability to escape from conditions that may ultimately lead to death, was determined using the dynamic method (Lutterschmidt and Hutchinson, 1997; Duarte et al., 2012). Briefly, tadpoles were exposed to a constant heating rate of 0.5°C min^{-1} in a waterbath (24–44.1°C) until they no longer responded to mechanical stimulation with blunt forceps. At this time they were immediately transferred to water at room temperature to allow recovery. CT_{max} measurements were non-fatal and all tadpoles recovered.

Statistical analysis

The number of days taken to reach development stage 35–37 was analysed using a Kruskal–Wallis test with *post hoc* multiple comparisons, as data could not be transformed to meet assumptions of normality. Morphometric and CT_{max} data were analysed using ANOVA. M_b was included as a covariate in the analysis of CT_{max} data. Where indicated, Tukey's *post hoc* pairwise analyses were used. Mortality was analysed using logistic regression.

Non-linear functions can be used to estimate TPCs by modelling parameters which describe their shape. By using these parameter estimates we can determine changes in the shape of TPCs in response to different thermal environments (Angilletta, 2006; Arrighi et al., 2013). For tadpole

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RMR and U_{max}, we used non-linear regression to fit the quadratic function, $y=ax^2+bx+c$, to our data to describe TPCs through three parameters: a, the curvature of the apex; b, the slope; and c, the y-intercept. Other non-linear functions often used for TPCs (i.e. Weibull, Gaussian; Angilletta, 2009) were unable to be fitted to our data. Treatment and test temperature were included as fixed effects in these models as well as M_b and TL as covariates for RMR and U_{max}, respectively.

Enzyme activity was analysed using linear mixed-effects models with weighted residuals. Treatment and test temperature were included as fixed effects and tadpole ID was included as a random factor. Data were log or square root transformed where necessary and a polynomial term was included to test for curvature. If the polynomial term was significant, 95% confidence intervals were used to examine the effect of treatment. All analyses were done using the R statistical software package (R Development Core Team, 2014). Data are presented as means±s.e.m.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
P.K., R.L.C. and C.E.F. designed the experiment; C.E.F. provided materials; P.K. performed experiments, analysed the data and wrote the manuscript; C.E.F. and R.L.C. revised the manuscript.

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Supplementary material
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