Physiological responses of ectotherms to daily temperature variation

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Abstract

Daily thermal fluctuations (DTF) impact the capacity of ectotherms to maintain performance and energetic demands due to thermodynamic effects on physiological processes. Mechanisms which reduce the thermal sensitivity of physiological traits may buffer ectotherms from the consequences of DTF. Species which experience varying degrees of DTF 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 DTF tadpoles from habitats characterised by small DTF would show greater plasticity in the thermal sensitivity physiological processes than tadpoles from environments characterised by large DTF. We tested the thermal sensitivity of physiological traits in tadpoles of three species which differ naturally in their exposure to DTF raised in control (24°C) and DTF treatments (20-30°C and 18-38°C). DTF reduced growth in all species. Development of tadpoles experiencing DTF 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 DTF induced plasticity in metabolic enzyme activity in all species, although to a greater extent in species from less thermally variable environments. DTF increased upper thermal limits in all species (between 0.89-1.6°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 likely 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 due to thermodynamic effects on biochemical reaction, 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 (DTF) are particularly challenging for ectotherms. Due to 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, DTF increase metabolic demands compared to constant temperature conditions, causing energy trade-offs that can effect 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 DTF can increase energetic demands which result in decreased rates of development (Niehaus et al., 2006; Dhillon and Fox, 2007; Les et al., 2007), and reduced body size at maturity compared to 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, DTF can increase body size and rate of development (Dong et al., 2006; Du and Feng, 2008; Folguera et al., 2011) as responses to DTF are highly variable between species/traits.

Ectotherms exposed to DTF 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 DTF 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 DTF (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
DTF experienced in different microclimates (Williams et al., 2012). In response to DTF, 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 DTF (Niehaus et al., 2011; Kern et al., 2014).

As with other forms of plasticity, what drives response to DTF may be the degree of thermal variability an organism experiences in their environment (Relyea, 2002; Gabriel et al., 2005). Ectotherms from environments with little diurnal temperature variation would benefit from mechanisms which reduce the thermal sensitivity of physiological traits in response to increased DTF, in order to reduce metabolic costs of exposure to high temperatures (Sinclair et al., 2006; Williams et al., 2012). Ectotherms from environments characterised by large DTF may already have thermally insensitive physiological rates due to selection pressures of a highly variable environment (Huey and Hertz, 1984). As such, their performance breadth may already span the range of environmental temperatures experienced, limiting the benefits of plasticity in response to DTF (Williams et al., 2012). When DTF extend beyond the normal range of temperature fluctuations experienced, animals with the capacity to reduce their thermal sensitivity may be more robust than non-plastic phenotypes. Determining what shapes physiological responses to DTF may identify species which are capable at 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 response to DTF, 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 related species of Australian frogs, whose developmental environments vary in the degree of DTF due to 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 thermal sensitivity of physiological processes in response to DTF than tadpoles from highly thermally variable environments. This comparison may allow us to understand whether environmental variability determines physiological responses to DTF.
Results

Water temperatures

Water temperatures of shallow pools at *P. ornatum* collection sites had a mean of 24.7 ± 0.3°C, and average daily fluctuations of 17.5 ± 0.3 °C. Deep pools at the same geographical location where *L. tasmaniensis* eggs were collected had a mean of 23.9 ± 0.2°C, and average daily fluctuations of 13.2 ± 0.1°C. Water temperatures 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 tadpoles in the control treatment (39 %; $c^2(2, N = 340) = 12.79 < 0.001$). 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 %).

Time taken to reach development stage 35-37 was longer for tadpoles in the small DTF treatment compared to control treatment for *Limnodynastes* species (*L. peronii* $c^2(1, N = 192) = 135.11 p < 0.001$; *L. tasmaniensis* $c^2(1, N = 163) = 118.97 p < 0.001$). Conversely, development was shorter for *P. ornatum* tadpoles in both fluctuating treatments compared to the control ($c^2(2, N = 245) = 32.86 p < 0.001$). For *P. ornatum* tadpoles the time to reach development stage 35-37 was not different between the small and large DTF treatments (Table 1).
Table 1. Morphometrics and time taken to reach development stage 35-37 of tadpoles raised in control, small DTF and large DTF treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Development* (days)</th>
<th>Body mass (g)</th>
<th>Body length (mm)</th>
<th>Tail length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. peronii</td>
<td>24°C</td>
<td>90.79 ± 0.72q</td>
<td>338.24 ± 7.41</td>
<td>12.44 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>20-30°C</td>
<td>115.55 ± 0.86b</td>
<td>320.95 ± 5.4</td>
<td>12.46 ± 0.1</td>
</tr>
<tr>
<td>L. tasmaniensis</td>
<td>24°C</td>
<td>156.43 ± 1.1e</td>
<td>463.13 ± 8.92g</td>
<td>14.16 ± 0.12i</td>
</tr>
<tr>
<td></td>
<td>20-30°C</td>
<td>200.93 ± 0.65f</td>
<td>370.12 ± 9.24b</td>
<td>13.46 ± 0.13j</td>
</tr>
<tr>
<td>P. ornatum</td>
<td>24°C</td>
<td>29.75 ± 0.38m</td>
<td>243.26 ± 6.16o</td>
<td>11.48 ± 0.12r</td>
</tr>
<tr>
<td></td>
<td>20-30°C</td>
<td>25.60 ± 0.33a</td>
<td>182.66 ± 4.62p</td>
<td>10.46 ± 0.11s</td>
</tr>
<tr>
<td></td>
<td>18-38°C</td>
<td>25.74 ± 0.46a</td>
<td>140.45 ± 3.6q</td>
<td>9.67 ± 0.09t</td>
</tr>
</tbody>
</table>

*Time taken to reach development stage 35-37

Letters denote significant differences

Body mass and BL of L. peronii tadpoles was not significantly affected by treatment, whereas TL was significantly reduced by small DTF (Table 1; TL F\_1,188 = 8.27, p = 0.005). For both L. tasmaniensis and P. ornatum Mb, BL and TL were all significantly reduced in tadpoles in small DTF (Table 1; L. tasmaniensis logMb 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 sqrtMb 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 DTF (TukeyHSD all comparisons p < 0.05).

**Maximum Burst swimming performance (Umax)**

Maximum burst swimming performance 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 (RMR)**

The small DTF treatment significantly affected the TPC for RMR of L. tasmaniensis through the shape of the apex (a), the slope (b), and the y-intercept (c) (Table 2). This resulted in reduced thermal
sensitivity at low temperatures, and increased metabolic rate at the highest test temperature compared
to 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*.

Table 2 ANOVA table from quadratic regression on RMR data. There was a significant effect of
treatment and test temperature on RMR in *L. tasmaniensis*.

<table>
<thead>
<tr>
<th></th>
<th>A d.f</th>
<th>F-value</th>
<th>p-value</th>
<th>d.f</th>
<th>F-value</th>
<th>p-value</th>
<th>d.f</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test temperature</td>
<td>1.74</td>
<td>0.93</td>
<td>0.34</td>
<td>1.74</td>
<td>2.99</td>
<td>0.09</td>
<td>1.74</td>
<td>26.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.74</td>
<td>4.85</td>
<td>0.03</td>
<td>1.74</td>
<td>5.07</td>
<td>0.03</td>
<td>1.74</td>
<td>5.84</td>
<td>0.02</td>
</tr>
</tbody>
</table>

RMR, resting metabolic rate.

Data are for parameters from the function: *y*=*ax*²+*bx+c*.

**Metabolic enzyme activity**

In *L. peronii* tadpoles, the activities of LDH and CS enzymes were significantly higher in tadpoles
from the small DTF treatment than tadpoles from the control treatment (Fig 4; supplementary
material). 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). Activity of CS increased with test temperature
(*F*$_{1,109}$ = 1744.04 *p* < 0.001), and was significantly higher in tadpoles from small DTF compared to
tadpoles from the control (*F*$_{1,21}$ = 6.07 *p* = 0.02). Activity of CCO was significantly affected by test
temperature (*F*$_{1,118}$ = 401.75 *p* <0.001), but was not significantly different between treatments (Fig 4a-
c).

In *L. tasmaniensis* tadpoles, the activity of all metabolic enzymes was higher in tadpoles from the
small DTF treatment compared to tadpoles from the control treatment (Fig 4). 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 to tadpoles from the control (*F*$_{1,22}$ = 6.72 *p* = 0.02; Fig 4d-f).

Between treatments, activity of LDH and CCO was affected by an interaction between treatment and
the polynomial term (LDH *F*$_{2,116}$ = 72.77 *p* <0.001, CCO *F*$_{2,116}$ = 7.45 *p* <0.001) inferring different rate
curves in response to temperature change. LDH activity was significantly higher at high temperatures
in tadpoles in the small DTF treatment, while CCO activity was significantly higher in tadpoles from
the small DTF treatment across the range of temperatures (supplementary material).
In *P. ornatum* tadpoles only the activity of CS was significantly different between treatments. CS activity was significantly lower in tadpoles from the large DTF compared to the control and small DTF, and CS activity in the small DTF treatment was significantly lower than control between 18-33°C test temperatures. Activity of CS and CCO was affected by an interaction between treatment and the polynomial term (CS $F_{4,154} = 97.83, p < 0.001$, CCO $F_{4,154} = 6.20, p < 0.001$). There was no effect of treatment on CCO activity (Fig 4g-i; supplementary material). In tadpoles from the control and large DTF treatments, activity of LDH increased linearly with increasing test temperature, while LDH activity of tadpoles in the small DTF 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.

**CTmax**

Tadpoles of all three species in small DTF had significantly higher CTmax 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* $F_{2,16} = 37.80, p < 0.001$). CTmax for *P. ornatum* tadpoles was also significantly higher in tadpoles in the large DTF compared to control treatment (TukeyHSD $p < 0.001$) but there was no difference between the CTmax of tadpoles that experienced small and large DTF. *P. 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 DTF had reduced growth compared to tadpoles in constant temperature in all species. Increased energy demands are an important consequence of short-term temperature variation due to 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). DTF 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 DTF compared to those in constant temperatures, 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 DTF. 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 which indicate drying of their habitat (Newman, 1989; Szekely 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; Szekely et al., 2010). This allows larvae to reach metamorphosis before their habitat desiccates. DTF 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 unable to increase development in response to DTF and metamorphose before habitats dry.

Different consequences of DTF 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 DTF on the length of development.

All species showed limited responses to DTF 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 DTF. In response to small DTF, *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 temperatures, however, was coupled with greater thermal sensitivity at high temperatures compared to 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 DTF in this species. Reduced thermal sensitivity at high or low temperatures in response to DTF 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 DTF.

Daily fluctuating temperatures 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 DTF. *L. peronii* and *L.
*tasmaniensis* tadpoles increased aerobic metabolic enzyme activity in response to small DTF (CS and CCO and CS, respectively) however this was not reflected by RMR. Therefore, changes in metabolic enzyme activity do not directly correlate to changes in metabolism. Resting metabolic rate indicates maintenance costs whereas the activity of CCO and CS represent 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 activities in *Limnodynastes* species may provide a greater metabolic scope that would help meet metabolic demands for growth in a thermally variable environment. Lactate dehydrogenase 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 DTF 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 likely 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 DTF.

Altered metabolic enzyme activity in response to DTF shows that DTF did provide a cue for plasticity in these traits. As *Limnodynastes* species increased enzyme activity rather than decreasing the thermal sensitivity of this trait as predicted, it is worth considering what aspect of DTF acted as a cue for plasticity. The increase in enzyme activity in response to small DTF 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.

Daily thermal fluctuations induced plasticity in the upper thermal limits of all species. Tadpoles exposed to DTF had higher upper thermal limits than those in constant temperature conditions. Plasticity of temperature tolerance in response to DTF 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 timescale (Seebacher et al., 2012). *L. peronii* and *L. tasmaniensis* are closely related yet experience a greater difference in environmental temperatures than *L. tasmaniensis* and *P. ornatum*. Phylogenetic relatedness may therefore explain similar changes in enzyme activities in response to DTF. In theory, the capacity for plasticity is determined by the amount of environmental variation 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*. On the other hand, 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 DTF in the environments 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 DTF 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 DTF 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; Kjaersgaard et al., 2013; Kern et al., 2014). In this study, DTF increased upper thermal limits which may buffer tadpoles from lethal consequences of temperature extremes. However the inability to buffer metabolism from DTF meant that growth and development (in *Limnodynastes* species) were negatively impacted. Importantly, different species exhibit different responses to DTF 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 likely 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 DTF in tadpoles of three species which develop in different thermal environments; *Limnodynastes peronii* (Duméril & 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. *P. ornatum* inhabit dry environments and breed after heavy rain in highly ephemeral water bodies characterised by large DTF (>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). *L. peronii* and *L. tasmaniensis* are usually associated with permanent and semi-permanent water bodies that experience less DTF (Fig 1). These species can breed successfully in a range of habitats from permanent dams and lakes, and ephemeral flooded grasslands and pools (Anstis, 2002). *L. peronii* tadpoles have the capacity to acclimate to stable temperatures and have been shown to have thermally sensitive TPC in 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, Queensland, Australia (27°19’ S, 151°05’ E). Eggs of the former were found in deeper water bodies, while the latter were found in very shallow pools (pers. observation). Three partial clutches of *L. peronii* eggs were collected from water bodies (collected in May) in St Lucia, Queensland, Australia (27°30’ S, 152°59’ E). After collection, egg masses were transported to The University of Queensland. Water temperatures at a depth of ~10cm were recorded at collection sites every hour for one month (iButton, Maxim Integrated Products Inc., San Jose, CA, USA).
Experimental treatments

Eggs were separated and placed individually into 80ml containers in chemically aged water (Prime, Seachem, Georgia, USA). Tadpoles developed in one of three temperature treatments (each had a mean of 24°C); control (24°C), small DTF (20-30°C) and large DTF (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 in 14:10 (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 hind limbs 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 for either critical thermal maximum (CTmax), or at one of six temperatures for resting metabolic rate (RMR) or maximum burst swimming performance (Umax). We then recorded body mass (M_b, in g), body length and tail length (BL and TL respectively, in mm) and number of days to reach development stage 35-37. After testing, tadpoles were euthanised by exposure to Aqui-S (175mg/L 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 (%).

Maximum burst swimming performance (Umax)

Maximum burst swimming performance 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. L. tasmaniensis was tested at five temperatures (18, 23, 28, 33, and 38°C). Tadpoles were removed from temperature treatments when temperatures were ~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 hour. Burst swimming performance was assessed in a swimming arena (27 x 13 x 5 cm) lined with reflective tape to give a clear silhouette of each tadpole. This container was filled with dechlorinated aged tap water to 3 cm deep to prevent vertical movement, and semi-submerged in a water bath set to 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 degree 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 maximum burst performance (Umax).

**Resting metabolic rate (RMR)**

Resting metabolic rate 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*. *L. 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 water bath 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 water bath allowed for the correction of O₂ solubilities with changing water temperature.

Oxygen consumption (VO₂; mL O₂ h⁻¹) was calculated using the following formula:

\[ VO₂ = (ΔO₂ \times V)/T \]

Where ΔO₂ is the change in oxygen in the chamber (mLO₂L⁻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, lactate dehydrogenase (LDH), and two enzymes involved in aerobic respiration, cytochrome C oxidase (CCO) and citrate synthase (CS). We used tail muscle dissected from tadpoles used 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 CCO, 1:100 LDH; 50mM imidazole, 2mM MgSO₄, 5mM EDTA, 0.1%
Triton X-100 and 1mM glutathione, pH = 7.5) and enzyme assays 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.

**Critical thermal maximum (CTmax)**

CTmax, 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 per minute in a water bath (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. CTmax measures were non-fatal and all tadpoles recovered.

**Statistical Analysis**

Number of days taken to reach development stage 35-37 was analysed using Kruskal Wallis test with post-hoc multiple comparisons, as data could not be transformed to meet assumptions of normality. Morphometric and CTmax data were analysed using ANOVA. Where indicated, Tukey’s post-hoc pairwise analyses were used. Mortality was analysed using logistic regression.

Non-linear functions can be used to estimate TPC by modelling parameters which describe their shape. By using these parameter estimates we can determine changes in the shape of TPC in response to different thermal environments (Angilletta, 2006; Arrighi et al., 2013). For tadpole RMR and maximum burst swimming performance we used non-linear regression to fit data to the quadratic function, \( y = ax^2 + bx + c \), to describe TPC through three parameters: \( a \): curvature of the apex, \( b \): slope and \( c \): y-intercept. Other non-linear functions often used for TPC (i.e. Weibull, Gaussian; Angilletta, 2009) were unable to be fit 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 burst swimming performance 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 sqrt- 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 mean ± s.e.m.

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References


Figure 1. Habitat temperatures of study species and treatment temperatures. Water temperatures at a depth of ~10 cm were recorded every half hour for one month at collection sites of (a) *L. peronii*, (b) *L. tasmaniensis* and (c) *P. ornatum*. Black lines represent daily mean temperatures, while dashed lines represent the daily maximum (above) and daily minimum (below) temperatures. (d) Treatment temperatures reflect habitat temperatures; control treatment (24°C; blue), small DTF (20-30°C; red) and large DTF (18-38°C; green) treatments.
Figure 2. Maximum burst swimming performance of tadpoles raised in control and fluctuating temperatures. Maximum burst swimming performance (mean ± s.e.m) of tadpoles was tested at 13, 18, 23, 28, 33, 36/38 °C. Swimming performance of (a) *L. peronii*, (b) *L. tasmaniensis* and (c) *P. ornatum* tadpoles was not different between control (24°C; blue), small DTF (20-30°C; red) and large DTF (18-38°C; green) treatments.
Figure 3. Oxygen consumption of tadpoles raised in control and fluctuating temperatures. Rate of oxygen consumption (mean ± s.e.m) of tadpoles was tested at 13, 18, 23, 28, 33 and 36/38°C. Oxygen consumption 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 temperatures for tadpoles raised in small DTF (20-30°C; red) compared to tadpoles raised at control temperatures (24°C; blue).
Figure 4a-i. Metabolic enzyme activity of tadpoles raised in control and fluctuating temperatures. Enzyme activity of lactate dehydrogenase (LDH), citrate synthase (CS) and cytochrome C oxidase (CCO) for tadpoles of *L. peronii* (a-c), *L. tasmaniensis* (d-f) and *P. ornatum* (g-i) tadpoles raised in constant (24°C; blue), small DTF (20-30°C; red) and large DTF (18-38°C; green), tested at 13, 18, 23, 28, 33 and 38°C.
Figure 5. Critical thermal maxima (CTmax) of tadpoles raised in stable and fluctuating temperatures. CTmax 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; red) and large DTF (18-38°C; green) compared with tadpoles in the control temperature (24°C; blue). Asterisk denote significant differences between treatments.