Physiological responses to daily temperature variation and ultraviolet radiation in amphibian larvae

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B An Vet Bio Sci (Hons 1)

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ABSTRACT

Understanding how environmental variability influences species fitness is important for predicting species persistence in changing environments. Environments are highly variable and organisms have discrete physiological limits which determine the range of conditions they can tolerate. Daily thermal fluctuations (DTFs) impact the capacity of ectotherms to maintain performance and energetic demands due to thermodynamic effects on physiological rates. However, organisms can also flexibly alter their phenotype in order to maintain fitness in variable environments. Ectotherms experiencing DTFs would benefit from mechanisms which reduce the thermal sensitivity of physiological traits and buffer energetic costs. Furthermore, energetic consequences of DTFs may be exacerbated by the presence of additional stressors. Exposure to ultraviolet radiation (UVR) can increase energy requirements due to the repair of UVR induced damage. The costs of UVR exposure may cause energy trade-offs which exacerbate consequences of DTFs, and reduce the capacity to flexibly alter phenotypes. Importantly, temperature and UVR are also linked in their effects on cellular function as exposure to temperature extremes and UVR causes cellular damage that induces common protective mechanisms. Interactive effects of these environmental drivers on mechanistic traits may explain whole animal responses. Understanding what drives responses to temperature variation and the interactive effects between environmental stressors will broaden our understanding of how animals respond to environmental variation. The overarching aim of this thesis was to investigate physiological mechanisms underlying responses to DTFs and exposure to UVR in amphibian larvae.

The capacity to flexibly alter the thermal sensitivity of traits in response to DTFs may depend on the degree of environmental DTF species experience. The first specific aim of this thesis was to investigate whether plasticity in response to DTFs is influenced by the thermal variability of a species’ habitat (Chapter 2). Tadpoles of three related species whose habitats vary in the degree of DTF were raised in stable temperatures and conditions in which temperatures fluctuated daily. Plasticity of upper thermal limits and the thermal sensitivity of swimming performance, resting metabolic rate and metabolic enzyme activity were examined. Environmental variation in species habitats did not predict the capacity to alter physiological rate processes. Tadpoles were unable to reduce the thermal sensitivity of physiology traits, which led to smaller body sizes and altered the length of development in a species specific fashion. DTFs increased upper thermal limits which may
buffer tadpoles from the lethal consequences of temperature extremes. The effects of DTFs on growth and development however, will likely negatively impact individual fitness.

The second specific aim of this thesis was to investigate whether UVR exposure alters the physiological response to DTFs (Chapter 3). Tadpoles were raised in stable or daily fluctuating temperature conditions under high or low UVR levels, and the plasticity of upper thermal limits and the thermal sensitivity of swimming performance and resting metabolic rate were examined. Temperature and UVR exposure had an interactive effect, with DTFs reducing survival and increasing upper thermal limits only in tadpoles exposed to UVR. Tadpoles (*Platyletrum ornatum*) which inhabit ephemeral pools characterised by large DTFs and high UVR were inherently thermally insensitive for burst swimming performance and metabolic rate at high temperatures, and these traits were not affected by temperature or UVR treatments. Inherent thermal insensitivity may buffer development from the energetic challenges of such variable environments.

Interactive effects of temperature and UVR exposure may reflect responses of lower level mechanistic traits. The third specific aim of this thesis was to investigate the interactive effects of temperature and UVR on upper thermal limits, heat shock protein (Hsp) abundance, antioxidant activity and oxidative damage (Chapter 4). Tadpoles were exposed to cold, warm or daily fluctuating temperature treatments in the presence or absence of UVR. Upper thermal limits were determined by an interaction between temperature and UVR. Ultraviolet radiation increased upper thermal limits in cold acclimated tadpoles and reduced upper thermal limits in warm acclimated tadpoles. This interactive effect was not explained by the relative abundance of Hsp70. Oxidative damage and antioxidant activity were influenced by UVR and temperature respectively, however there were no interactive effects. Understanding the interactive effects of multiple stressors on thermal tolerance is important to predict responses to environmental variation. Thermal tolerance is however, a complex trait and may not be readily explained by a subset of molecular and cellular mechanisms.

This research has contributed to a broader understanding of the physiological consequences of DTFs and UVR in amphibian larvae. The results highlight that responses to DTFs may be species specific which may influence survival in variable environments, and that consequences of DTFs are exacerbated by exposure to UVR. Importantly, interactive effects between stressors determined physiological limits. Responses to these
environmental drivers are complex and likely determined by interactions among a range of mechanistic traits. Physiological responses to different scales of temperature variation and the interactive effects of multiple stressors determine how animals function, and may ultimately determine population persistence in changing environments.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Contributions by others to the thesis

Craig Franklin and Rebecca Cramp, through discussions with myself, contributed significantly to the conception and design of this research overall. Frank Seebacher edited and provided important feedback on Chapter 4. Ensiyeh Ghanizadeh Kazerouni provided significant technical assistance in Chapter 4. Simon Blomberg provided important assistance with statistical analysis of Chapter 2 and 3. Julian Beaman provided feedback on all chapters of this thesis. Craig Franklin and Rebecca Cramp critically reviewed the final draft of this thesis.

Statement of parts of the thesis submitted to qualify for the award of another degree

None
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Ok, next adventure.
KEYWORDS

Tadpole, daily thermal fluctuation, interactive effects, performance, metabolism, critical thermal maximum, heat shock protein, oxidative damage

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LIST OF ABBREVIATIONS

BL – body length
COX – cytochrome c oxidase
CS – citrate synthase
CSR – cellular stress response
CT\textsubscript{max} – critical thermal maximum
CT\textsubscript{min} – critical thermal minimum
DTF – daily thermal fluctuation
Hsp – heat shock protein
HSR – heat shock response
LDH – lactose dehydrogenase
M\textsubscript{b} – body mass
MDA – malondialdehyde
RMR – resting metabolic rate
ROS – reactive oxygen species
TBA – thiobarbituric acid
TL – tail length
T\textsubscript{o} – thermal optimum
TPC – thermal performance curve
U_{\text{max}} – maximum burst swimming performance
UVR – ultraviolet radiation
CHAPTER 1

GENERAL INTRODUCTION

Environmental variation

Environmental variation is an important driver of species abundance and distribution, and ecosystem functioning. Environments vary in temperature, exposure to sunlight, predator density, oxygen availability and many other variables along both spatial and temporal scales. Organisms generally have discrete physiological limits which determine the range of conditions they can tolerate (Guderley, 2004; Hofmann and Todgham, 2010). Behavioural and physiological responses to environmental variability can ameliorate the consequences of environmental variation and influence the capacity of organisms to persist under changing conditions (Seebacher and Franklin, 2005; Seebacher et al., 2015). Predicting species responses to environmental variation requires an understanding of the physiological tolerances of individuals and their capacity to alter these tolerances (Deutsch et al., 2008; Sinervo et al., 2010; Duarte et al., 2012). Understanding the capacity for behavioural and physiological mechanisms to buffer animals from the consequences of variable environments will be important in predicting how environmental change will influence species persistence (Chevin et al., 2010).

Animals demonstrate flexible responses to a range of environmental factors by altering their behaviour, morphology and physiology, which allows them to maintain fitness in variable environments. For example, phenotypic flexibility allows animals to respond to temperature variation by altering the thermal sensitivities of enzymes in thermally variable environments (Seebacher et al., 2014), improving aerobic swimming performance in hypoxic conditions (Yang et al., 2013) and increasing salinity tolerance when acclimated to saline conditions (Wu et al., 2014). These responses buffer organisms from the energetic costs and physiological constraints of environmental variation by optimising physiological function in the new environment and increasing physiological tolerance to these conditions. The capacity to flexibly alter phenotypes in response to environmental perturbation is widespread, and can involve changes in behaviour, physiology and gene expression, which buffer fitness under new conditions (Wilson and Franklin, 1999; Podrabsky and Somero, 2004; Seebacher and Grigaltchik, 2014). While physiological adjustments are unlikely to fully overcome the consequences of environmental variation,
the capacity to mitigate the associated costs may be important in determining species persistence in the future (Seebacher et al., 2015). Temperature and ultraviolet radiation (UVR) are key environmental drivers. Determining how variation in these drivers affects animal physiology will increase our ability to predict population responses to environmental change. This thesis will examine the effects of temperature variation and exposure to UVR on animal physiology, and the interactive effects that arise from simultaneous exposure to these stressors.

Consequences of temperature on ectotherm physiology

Temperature has a pervasive influence on organism physiology. Thermodynamic effects on biochemical reactions alter growth, reproduction and performance (Hochachka and Somero, 2002). Numerous important cellular properties and physiological traits are temperature sensitive, including the composition of membranes, enzyme activities, muscle function, digestion, and aerobic respiration (Somero, 1978; Harwood, 1979; Hochachka and Somero, 2002; Seebacher et al., 2014). Aerobic respiration is the major process for metabolic energy production in vertebrates (Hochachka and Somero, 2002), and as such temperature variability can substantially influence energy balance (Niehaus et al., 2006; Arrighi et al., 2013). Energetic consequences of environmental temperature variation are especially pertinent for ectotherms whose body temperature reflect ambient conditions.

Temperature can vary spatially in the environment and during the lifetime of an individual on a timescale from minutes to seasons, and with major shifts in climatic patterns for longer lived organisms. Environments can differ in the degree of temperature variation and the rate of temperature change. For example, Antarctic waters are extremely thermally stable environments varying annually by only ~1.3°C (Seebacher et al., 2005). More commonly however, annual cycles lead to seasonal differences in temperature with the degree of seasonal variation generally increasing from low to high latitudes (Conover, 1992; Yamahira and Conover, 2002). Environments also experience more rapid rates of temperature change due to intra-seasonal weather patterns and regular daily (diurnal) fluctuations in temperature (Smith, 1972; Larkin and Harrison, 2005). Most environments experience at least some degree of diurnal temperature shift (Smith, 1972; Scholnick, 1994; Podrabsky and Somero, 2004; Williams et al., 2012; Seebacher et al., 2014), which can be as great as seasonal variation in some environments (Morris and Taylor, 1983). Anthropogenic climate change is causing both an increase in the Earth’s annual mean
surface temperatures as well as more frequent hot and fewer cold temperature extremes on both daily and seasonal timescales (IPCC, 2013). Understanding how animals cope with all levels of temperature variation is therefore crucial in order to predict the impact of climate change on species persistence.

Organisms typically exhibit discrete thermal limits which define the range of temperatures they can tolerate. Within this range of thermal tolerance, the thermal sensitivity of traits determines how thermal variability influences physiology. The thermal sensitivity of traits is often illustrated using a thermal performance curve (TPC, Figure 1.1, Angilletta, 2006). TPCs are generally concave in shape bounded by upper and lower thermal limits (critical thermal maximum [CT\text{max}], and minimum [CT\text{min}]) which define the organism’s performance breadth. The peak of the curve represents the thermal optima (T_0), the temperature at which performance/physiological function is maximised. TPCs illustrate how a trait (i.e. metabolism, growth, performance) will be affected by acute changes in temperature, and how environmental temperature variability might influence mean trait values. Non-linear equations can be used to estimate parameters which describe the thermal sensitivity of the TPC (Peek et al., 2002) and to illustrate how a trait changes as a consequence of the thermal conditions experienced (Angilletta, 2006).

![Figure 1.1](image-url)  
**Figure 1.1.** A hypothetical thermal performance curve (TPC) showing the performance breadth, upper and lower thermal limits (CT\text{max} and CT\text{min} respectively) and the thermal optimum (T_0).
Environments characterised by large, short-term temperature variation such as tidal mudflats and ephemeral pools, can experience temperature variation >20°C over 24 hours (Chapter 3; Tomanek and Somero; 1999; Podrabsky et al., 2008). The non-linear relationship between temperature and many physiological processes such as metabolism, enzyme activities, digestion and muscle function result in increased energy requirements for cell maintenance at high temperatures (Ruel and Ayres, 1999; Hochachka and Somero, 2002). Daily thermal fluctuations (DTFs) are therefore particularly energetically challenging for ectotherms as the time spent at high temperatures increases energetic demands (Ruel and Ayres, 1999; Hochachka and Somero, 2002). As such ectotherms exposed to DTFs often have higher energetic costs than those experiencing the equivalent mean temperature (Niehaus et al., 2012; Arrighi et al., 2013). DTFs can increase energetic demands during development leading to reduced body size compared to animals developing at the equivalent mean temperature (Atkinson, 1996; Dong et al., 2006; Niehaus et al., 2006; Dhillon and Fox, 2007) although not always (Du and Shine, 2010; Folguera et al., 2011). Energetic consequences of DTFs can also increase or decrease rates of development (Chapter 2; Dhillon and Fox 2007; Les et al., 2007; Folguera et al., 2011). The diverse responses to DTFs are likely associated with the specific thermal sensitivity of the traits and species investigated.

Fluctuating temperatures have complex effects on mean trait values when the temperature-trait relationship is described by a non-linear function (Jensen’s inequality, Ruel and Ayres, 1999). For example consider a trait, locomotor performance that has a non-linear relationship with temperature (Figure 1.2). When temperature increases performance with an accelerating function (convex), temperatures above the mean will increase performance more than temperatures below the mean decrease performance. Therefore when temperatures fluctuate, average performance (the mean trait value) will be greater than when experiencing constant mean temperature conditions (Figure 1.2A). The opposite is true when temperature increases performance with a decelerating (concave) function, where time spent below the mean temperature decreases performance more than time spent above the mean temperature increases performance. In this case temperature fluctuations will decrease the mean trait value (Figure 1.2B, Ruel and Ayres, 1999; Bozinovic et al. 2011). The response of trait values to DTFs will therefore depend upon the curvature of the temperature-trait relationship and the range of temperatures experienced. Without knowledge of the thermal sensitivity of traits, it is therefore difficult to predict the energetic consequences of DTFs.
Figure 1.2. Fluctuating temperatures can increase or decrease mean trait values compared to average temperature conditions due to the properties of non-linear functions described by Jensen’s inequality (Ruel and Ayres, 1999). Open circles represent mean temperatures and black arrows represent thermal fluctuations around the mean. When the trait function is accelerating (A), temperatures above the mean temperature increase performance more than temperatures below the mean temperature decrease performance (grey arrows), resulting in an increased mean performance compared to constant temperature conditions. The opposite is true when the trait function is decelerating (B). When the function is decelerating temperatures below the mean decrease performance more than temperatures above the mean increase performance, so that temperature fluctuations result in a lower mean performance value compared to mean temperature conditions.

**Physiological responses to temperature variation**

Many ectotherms have physiological mechanisms that can reduce the negative consequences of temperature variability. Physiological flexibility which alters the thermodynamic effects of temperature on trait values can buffer animals against the consequences of temperature variation. The capacity to reduce the energetic consequences of fluctuating temperatures may be important for coping with environmental variation and future shifts in environmental temperature (Seebacher et al., 2015).
Thermal phenotypic plasticity of physiological function and organism performance

Animals can alter their physiology in response to temperature variation in order to maintain physiological rates as conditions change. This phenotypic plasticity (acclimation/acclimatisation) allows individuals to reduce energetic demands and maintain performance in thermally variable environments by altering the shape or position of their TPCs (Angilletta, 2009). Phenotypic changes induced by the environment can become fixed if plasticity occurs during development (developmental plasticity), or can occur repeatedly throughout the life-time of an individual (reversible plasticity) as an acclimation response to changing environmental conditions (Gabriel et al., 2005; Angilletta, 2009). The magnitude, frequency and predictability of environmental variation determine the adaptive benefits of phenotypic plasticity (Gabriel et al., 2005; Angilletta, 2009; Botero et al., 2015). When environmental variation is larger between- than within-generations, selection should favour developmental plasticity. When the opposite is true, reversible plasticity should provide selective advantage (Levins, 1968; Angilletta, 2009). Phenotypic plasticity is induced by the environment in response to cues which provide information about present and future conditions (Levins, 1968; Gabriel et al., 2005). Acclimation should be beneficial when environmental variability is predictable through association with reliable cues (Levins, 1968; Johnston and Temple, 2002; Gabriel et al., 2005), and as such both seasonal variability and DTFs may be important for inducing plasticity (Sinclair et al., 2006; Williams et al., 2012; Seebacher et al., 2014).

Ectotherms experiencing large DTFs can benefit from plasticity which reduces the thermal sensitivity of physiological processes in order to buffer energetic demands by maintaining physiological and performance traits over the range of temperatures experienced. Plasticity in response to DTFs can reduce the thermal sensitivity of metabolism (Sastry, 1979; Williams et al., 2012), feeding filtration rates (Widdows, 1976) and swimming performance (Měráková and Gvoždík, 2009). However, when plasticity in response to DTFs is beneficial is difficult to predict, and some species/traits show no plasticity in response to these conditions (Henry and Houston, 1984; Niehaus et al., 2011). Short-term temperature fluctuations may mask signals for acclimation in some animals and/or their specific physiological traits. However short-term temperature variation does not always preclude acclimation responses to seasonal temperature variation (Seebacher et al., 2012). A reduction in the thermal sensitivity of traits in response to DTFs may be
associated with the lack of predictable cues in such variable environments (Botero et al., 2015) although this requires further investigation.

The benefits of plasticity in response to DTFs may be modulated by the degree of environmental thermal variability (Relyea, 2002; Gabriel et al., 2005). For example, ectotherms from environments that experience little short-term temperature variation may have a selective advantage from the capacity to flexibly alter the thermal sensitivity of their TPCs to reduce metabolic costs of novel DTFs (Sinclair et al., 2006; Williams et al., 2012; Seebacher et al., 2015). Alternatively, ectotherms inhabiting environments characterised by a high degree of DTFs may show canalisation for low thermal sensitivity and a wide performance breadth of physiological traits due to selection pressures of a highly variable environment (Huey and Hertz, 1984). This may limit their capacity to respond to DTFs if the breadth of their TPCs already span the range of temperatures experienced in their highly thermally variable environments (Sinclair et al., 2006; Williams et al., 2012).

Understanding what shapes physiological responses to DTFs will identify species that may be vulnerable to rapid, short-term temperature variation. Animals that show plasticity in response to high rates of temperature change may be robust at dealing with environmental perturbation.

**Plasticity of thermal tolerances**

Thermal tolerance defines the breadth of temperatures over which an individual can maintain physiological functions and performance. Thermal tolerances are maintained by natural selection, but are also plastic in response to the recent thermal history of an individual (Terblanche et al., 2010; Ravaux et al., 2012). In response to a heat or cold exposure, animals can alter upper or lower thermal tolerance limits (Cooper et al., 2012; Ravaux et al., 2012; McCann et al., 2014). Daily thermal fluctuations as well as increases in mean temperature can improve upper thermal limits in ectotherms (Feldmeth et al., 1974; Otto, 1974; Stillman, 2002; Schaefer and Ryan, 2006; Terblanche et al., 2010; Ravaux et al., 2012). The capacity to alter thermal limits can buffer individuals from damage caused by acute exposure to temperature extremes. Damage from temperature stress is attenuated by the induction of molecular chaperones which refold and prevent aggregation of denatured proteins (Dahlhoff, 2004). These mechanisms underlie thermal tolerance of organisms because they maintain cellular integrity under temperature stress (Feder and Hofmann, 1999). Maintenance of cellular function requires ATP for these
mechanistic traits (Dahlhoff, 2004). Diurnal fluctuations may therefore induce higher energetic demands than mean temperature conditions due to increased physiological rates and the costs of inducing protective cellular mechanisms at high temperatures.

Thermal limits indicate what temperatures an animal can tolerate, and as such they are important traits for understanding how species will respond to increasing environmental temperatures (Sinervo et al., 2010; Duarte et al., 2012). Species living at temperatures close to their upper thermal limits are thought to be at risk of extinction, or range reduction if they are unable to increase their thermal tolerance (Somero, 2010; Tomanek, 2010). Altering upper thermal limits may buffer organisms from increasing global temperatures, although only if the trait can adapt/evolve as rapidly as climate change will proceed (Sinervo et al., 2010). Incorporating plasticity of thermal tolerances in models of population persistence will more accurately determine the effects of changing environmental temperatures, and whether plasticity in upper thermal limits can buffer organisms from lethal effects of temperature extremes. Our ability to predict species persistence would therefore benefit from understanding what drives plasticity of upper thermal limits.

**Consequences of exposure to UVR on ectotherm physiology**

Like temperature, UVR (wavelengths between 100-400 nm; UV-A 100-300 nm, UV-B 300-320 nm and UV-C 320-400nm) is an important environmental factor which drives the ecology and evolution of individuals, populations and ecosystems (Cockell and Blaustein, 2001). Ultraviolet radiation levels vary both spatially and temporally due to natural, and in recent decades, anthropogenic factors. Variation in the atmospheric constituents which influence UVR transmission leads to unpredictability in the levels of UVR reaching the earth’s surface (Madronich, 1993). The presence of clouds, aerosols and local pollutants are also important in determining the transmission of UVR to the environment (Madronich, 1993; Herman, 2010). Over the last century, anthropogenic release of chlorofluorocarbons (CFCs) led to increased transmission of UVR to the Earth’s surface as a consequence of depletion of atmospheric ozone. Global action to phase out the use of ozone depleting substances are aiding in the recovery of the ozone, however UVR levels are predicted to remain high for a number of decades (Reddy et al., 2010).
Due to anthropogenic increases in UVR, it is important to understand whether exposure to this stressor causes energy trade-offs which alter the capacity of organisms to respond to temperature variation. Exposure to UVR causes cellular damage by distorting DNA, forming lesions, and structurally damaging proteins and lipids. This can lead to inefficiencies in transcription, disruption to structural and functional cellular components and cause mutations resulting in cell death (Tevini, 1993; Hessen, 2003; MacFadyn et al., 2004). Exposure to UVR can also indirectly damage cellular components due to the production of damaging reactive oxygen species (Fuchs and Packer, 1991). Most organisms can limit or mitigate the damage caused by UVR through behavioural avoidance, photo-protective compounds and UVR damage repair mechanisms (Kollias et al., 1991; Mitchell and Karentz, 1993; Palen et al., 2005). Maintaining photo-protective compounds, and or repairing UVR damage can increase energy requirements (Herbert and Emery, 1990; Sancar and Tang, 1993; Fischer et al., 2006), causing trade-offs which limit the energy available for other functions. Exposure to UVR has major consequences for organisms by increasing mortality (van Uitregt et al., 2007; Bancroft et al., 2008a) and causing a range of sub-lethal effects. Ultraviolet radiation exposure can cause energy partitioning which can reduce growth rates (Tevini, 1993; Caldwell et al., 1998; Bancroft et al., 2007), delay developmental rates (Blaustein et al., 1997; Croteau et al., 2008), and reduce locomotor ability (Blouin and Brown, 2000; Álvarez and Nicieza, 2002), activity levels (Alton et al., 2011) and immune function (Salo et al., 1998; Cramp et al., 2014). Furthermore the costs associated with exposure to UVR lead to energy trade-offs which often exacerbate negative consequences of additional stressors (Bancroft et al., 2008a).

**Interactive effects of temperature and UVR on ectotherm physiology**

The complexity of environments causes organisms to experience variation across different time scales, as well as variation in multiple environmental factors simultaneously. In environments characterised by large diurnal thermal variation, such as ephemeral aquatic environments, fluctuations in temperature associated with solar warming of the environments expose animals to peak environmental temperatures and high UVR simultaneously (Metaxas and Scheibling, 1993; Heilmeier et al., 2005). Exposure to multiple stressors can lead to energy partitioning where energy allocated to respond to one stressor reduces the energy available to respond to another (Martin et al., 2008; Searle et al., 2010; Applebaum et al., 2014). Multiple stressors acting in concert can produce
interactive effects on physiological function which determine animal responses to environmental variation.

The interactive effects of multiple stressors can be described using an additive effects model (Folt et al., 1999; Christensen et al., 2006). The effect of exposure to two (or more) stressors is additive when the consequence is equal to the sum of the effects of each stressor (Folt et al., 1999; Altshuler et al., 2011). An interactive effect arises when the consequence of exposure to multiple stressors is greater than (synergistic) or less than (antagonistic) the sum of the effects of the individual stressors (Folt et al., 1999). These interactive effects can be caused by energy trade-offs and/or cross-tolerance (hormesis) whereby exposure to a mild stress can upregulate mechanisms which protect against, or increase tolerance to additional stressors (Calabrese et al., 2007). The difficulty in predicting the outcome of exposure to multiple stressors dictates the need for multifactorial studies to better understand animal responses to environmental variation. For example, interactive effects of UVR with a range of abiotic/biotic stressors in *Limnodynastes peronii* tadpoles highlight the difficulty of predicting the effects of multiple stressors (Alton et al., 2010; Bernal et al., 2011; Mitchell et al., 2012). The consequence of hypoxia and UVR on tadpole hatching success and body mass is additive as the combined effects were equal to the sum of the effects of the stressors individually (Bernal et al., 2011). Exposure to predation risk and UVR, however, resulted in a synergistic interaction which increased tadpole mortality (Alton et al., 2010). Furthermore, conspecific density interacts antagonistically with UVR to mitigate negative consequences of reductions in body size (Mitchell et al., 2012). UVR can modulate the consequences of other stressors and as such the interactive effects of multiple stressors are complex and difficult to predict.

Temperature and UVR are major factors that independently shape the physiology of ectotherms. Importantly, temperature and UVR are also linked in their effects (Han et al., 2009; Cruces et al., 2013) and can interact synergistically to reduce fitness (Grant and Licht, 1995; Salo et al., 2000; van Uitregt et al., 2007; Moresino and Helbling, 2010; Cramp et al., 2014). Cold temperatures can exacerbate developmental abnormalities and mortality caused by exposure to UVR (Grant and Licht, 1995; van Uitregt et al., 2007; Moresino and Helbling, 2010). UVR induced DNA damage occurs independently of temperature (Pakker et al., 2000; Sanders et al., 2005). However, the major repair mechanisms are enzymatically mediated, and the thermal sensitivity of these repair mechanisms may explain the temperature dependent effects of exposure to UVR.
(Langenbacher et al., 1997; Pakker et al., 2000; MacFadyn et al., 2004; Sanders et al., 2005). For example, crabs reared at 20°C survived longer when exposed to UVR than those raised at 15°C (Moresino and Helbling, 2010) suggesting that protective mechanisms were better able to prevent or ameliorate the damage at warm temperatures. As enzyme activities are reduced at both extreme low and high temperatures (Somero, 1978), UVR damage may also be exacerbated during peak environmental temperatures due to thermodynamic effects on the activity of enzymatic repair mechanisms, although this has not yet been demonstrated (Alton et al., 2012).

**Cellular responses to temperature and UVR stress**

Environmental perturbation can initiate cellular protective mechanisms which mitigate damage caused by exposure to stress. Organisms respond to environmental stress through a widely conserved cellular stress response (CSR). The CSR involves the induction of a suite of genes/proteins that prevent and repair macromolecular damage and temporarily increases physiological tolerance to the imposed stress (Kultz, 2003, 2005). Initially the CSR results in the induction of heat shock proteins (Hsps). These molecular chaperones maintain the integrity of proteins during environmental stress by refolding denatured proteins and preventing the aggregation of non-native proteins through degradation (Lindquist and Craig, 1988; Iwama et al., 1998; Feder and Hofmann, 1999; Hartl and Hayer-Hartl, 2002). Hsps are constitutively expressed in cells, but also exist as stress-induced forms and are a suite of highly conserved protective mechanisms (Lindquist and Craig, 1988). Hsps are highly sensitive detectors of environmental stress during which they play a major role in preventing damage and temporarily increasing physiological tolerances (Sanders, 1993; Bierkens, 2000). Persistent cellular stress causes an alteration of pathways involved in proteolysis, cell growth, the cell cycle, protein degradation and metabolism (Cowan and Storey, 2003; Podrabsky and Somero, 2004). These changes result in a redistribution of energy to critical functions during stress, and plasticity in these responses determine the physiological tolerance of organisms to future stress events (Tomanek, 2010; Evans and Hofmann, 2012).

Temperature extremes and exposure to UVR can cause cellular damage directly, and indirectly through the production of reactive oxygen species (ROS, Fuchs and Packer, 1991; Abele et al., 2002). Damage caused by these stressors induces common protective mechanisms, and consequently the response to temperature stress may be moderated by
the presence of UVR. For example, exposure to high temperatures has been shown to increase resistance to subsequent UVR exposure through the activation of molecular chaperones (Trautinger et al., 1996). Exposure to both high temperature and UVR simultaneously may however overwhelm protective mechanisms resulting in increased cellular damage (Cruces et al., 2013). In order to understand responses to temperature and UVR exposure, it is essential to understand the interactive effects of these stressors on the mechanisms underlying physiological tolerances.

Temperature stress and exposure to UVR can cause cellular damage which initiates the heat shock response (HSR) and induction of Hsps. Exposure to high temperatures results in acclimation of the HSR which is associated with an increase in upper thermal limits (Tomanek and Somero, 1999; Tomanek and Somero, 2002; Fangue et al., 2011; Ravaux et al., 2012). This can involve an increase in constitutively expressed Hsps as well as plasticity in the induction temperature of stress inducible forms (Nakano and Iwama, 2002). Similar responses result from exposure to fluctuating temperatures, likely associated with the peak temperatures experienced (Feldmeth et al., 1974; Podrabsky and Somero, 2004; Todgham et al., 2006; Terblanche et al., 2010). Dose dependent increases in Hsps have also been associated with exposure to UVR (Kwon et al., 2002; Bonaventura et al., 2005; Bonaventura et al., 2006). Exposure to heat stress can reduce damage sustained by subsequent exposure to UVR, highlighting the role of Hsps in mitigating UVR induced damage (Trautinger et al., 1996). Such cross-tolerance or hormesis may result when exposure to one stressor induces Hsps which can improve the survival of cells and increase stress resistance of the organism (Arumugam et al., 2006). The role of Hsps in mitigating consequences of both temperature and UVR exposure may result in such interactive effects during simultaneous exposure. There is however, a deficiency of studies investigating the interactive effects of temperature and UVR on the expression of Hsps and determination of upper thermal limits.

High temperatures and exposure to UVR can indirectly increase oxidative damage to DNA, proteins and lipids through the production of ROS. Reactive oxygen species are highly chemically reactive molecules such as hydroxyl radical (\(\cdot OH\)), superoxide anion (\(O_2^-\)) and hydrogen peroxide (\(H_2O_2\)) which cause oxidation of cellular components often leading to apoptosis (Turrens, 2003). Reactive oxygen species are produced as a consequence of oxidative metabolism and are involved in cell signalling (Turrens, 2003; Apel and Hirt, 2004). Under normal conditions ROS are scavenged and broken down by antioxidants, but
under environmental stress increased ROS production can overwhelm antioxidant defences resulting in oxidative damage. As metabolism is temperature sensitive, higher temperatures can increase ROS production (Abele et al., 2002; Madeira et al., 2013) and absorption of UVR has been shown to initiate greater ROS production (Fuchs and Packer, 1991). As both high temperatures and exposure to UVR can increase the production of ROS, simultaneous exposure to both of these stressors may exacerbate oxidative damage.

Interactive effects of temperature and UVR on oxidative damage may be further complicated by thermodynamic influences on antioxidant defences. As many antioxidants are enzymes their activities are temperature sensitive (Kong et al., 2012). As such UVR-associated oxidative damage may be greater at low temperatures if antioxidant activity is impaired. At warm temperatures UVR induced ROS may be effectively scavenged due to increased activity of enzymatic antioxidants. However, extreme high temperatures may also impair antioxidant function (Somero, 1978). Antioxidant load and activity can acclimate in response to temperature (Selman et al., 2000; Vinagre et al., 2012; Meng et al., 2014) and UVR exposure can increase antioxidant activity in some cases (Han et al., 2009; Yu et al., 2009). This may result in complex interactive effects of temperature and UVR on antioxidant activity and oxidative damage. Interactive effects of temperature and UVR can exacerbate oxidative damage from UVR at high temperatures (Han et al., 2009; Cruces et al., 2013). However in some cases elevated temperatures can also elevate harmful effects of UVR (Han et al., 2009). Investigating the mechanistic cause of the interactive effects of temperature and UVR will be important for understanding what shapes animals responses to complex environmental variation.

Research framework

While some energetic consequences of DTFs have been investigated, our understanding of what drives physiological responses to these conditions is still developing. Determining when plasticity enables organisms to respond to DTFs will broaden our understanding of animal responses to temperature variation. Understanding developmental consequences of DTFs on individuals may identify species which benefit or are negatively impacted by short-term temperature variation. Furthermore, understanding responses to DTFs in the presence of an additional stressor (UVR) may highlight energy trade-offs which determine
consequences to environmental variation. The effects of temperature and UVR on the mechanistic traits which underlie physiological tolerance to these stressors may explain the interactive effects which occur at the whole animal level.

Species inhabiting semi-permanent and ephemeral fresh water ponds can experience high levels of DTF and UVR, as a consequence of the solar warming of these environments (Blaustein et al., 2001). Aquatic species are particularly susceptible to the consequences of environmental temperature variation, as body temperatures reflect ambient temperature due to high heat transfer rates in water (Duarte et al., 2012). Species inhabiting these environments may therefore experience consequences of DTFs on traits such as performance, growth and metabolic demands. Furthermore, behavioural preference for warm conditions can expose animals to UVR. For example, some tadpoles move to warmer water to facilitate growth and development, which exposes them to higher UVR (Bancroft et al., 2008b). As such, tadpoles inhabiting these environments provide a good model to investigate the interactive effects of DTFs and UVR. Tadpoles have been shown to thermally acclimate to stable temperatures (Wilson and Franklin, 1999; Seebacher and Grigaltchik, 2014) and are often used to investigate the consequences of UVR exposure due to high susceptibility to damage (Blaustein et al., 1998; Crump et al., 1999; Blaustein et al., 2001; Alton et al., 2011). Synergistic effects exacerbate consequences of UVR on growth, swimming performance and mortality in amphibian larvae when exposed to cold temperatures (Grant and Licht, 1995; van Uitregt et al., 2007).

**Aims of research**

The overall aim of this thesis was to investigate physiological responses to diurnal thermal fluctuations and exposure to UVR in anuran tadpoles. The tadpoles of three related anuran species (family Limnodynastinae, Schäuble et al., 2000) whose habitats vary in the amplitude of diurnal thermal variation were used to investigate what drives responses to DTFs and exposure to UVR.

Short-term temperature fluctuations are particularly energetically challenging for ectotherms, and reducing the thermal sensitivity of physiological processes may reduce the costs of these conditions. What determines the capacity to reduce the energetic consequences of short-term temperature fluctuations through phenotypic plasticity is not
yet understood. The first aim of this thesis was to investigate whether plasticity in response to DTFs is influenced by the thermal variability of species habitats (Chapter 2).

Energy trade-offs caused by additional environmental stressors may limit resources available for physiological responses to DTFs. Exposure to UVR causes cellular damage which can increase energetic demands. The second aim of this thesis was to investigate whether exposure to UVR altered the physiological responses to DTF by investigating consequences for growth, development, performance and metabolism (Chapter 3).

Exposure to high temperatures and UVR cause cellular damage which initiates common protective mechanisms and may lead to cross-tolerance between stressors. Furthermore, the repair of UVR induced damage is temperature sensitive. As such, simultaneous exposure to temperature and UVR may interact to alter physiological tolerance due to complex effects on cellular mechanisms. The third aim of this thesis was to investigate the interactive effects of temperature and UVR on upper thermal limits, Hsp abundance, antioxidant activity and oxidative damage (Chapter 4).

**Study species**

*Limnodynastes peronii*

The striped-marsh frog (*Limnodynastes peronii*) is commonly found along the east coast of Australia, where they occur over a wide range of latitudes between 16° and 42° (Wilson, 2001). Populations range from far north Queensland to southern Victoria, with populations in south-east South Australia and north-west Tasmania. Frogs call and breed throughout most of the year when conditions are appropriate, especially in the northern part of their distribution. Eggs are laid in a variety of permanent and semi-permanent water bodies from dams and garden ponds, to flooded grasslands and still stream ponds (Anstis, 2013). Tadpoles of *L. peronii* can thermally acclimate swimming performance in response to stable temperatures (Wilson and Franklin, 1999). Larvae of this species are also acutely sensitive to UVR exposure (van Uitregt et al., 2007; Alton et al., 2011; Alton et al., 2012).
**Limnodynastes tasmaniensis**

Spotted marsh frogs (*Limnodynastes tasmaniensis*) occur across most of eastern Australia, from north Queensland to Tasmania. Populations of this species have been shown to be highly eurythermal (Whitehead et al., 1989). They are associated with temporary or permanent water such as ponds, ephemeral pools and flooded ditches and grasslands. This species breeds opportunistically after heavy rains (Anstis, 2013).

**Platyplectrum ornatum**

The ornate burrowing frog (*Platyplectrum ornatum*) is commonly distributed across the top of Australia and along the east coast to mid-NSW. These frogs breed after heavy rainfall in ephemeral pools that can experience large diurnal thermal fluctuations >20°C (Chapter 3). Development in this species is very rapid to enable them to develop in highly ephemeral environments (Anstis, 2013).

**Structure of thesis**

This thesis comprises three experimental chapters (Chapters 2-4) that investigate physiological responses of amphibian larvae to DTF and UV exposure. The first experimental chapter (Chapter 2) investigates the capacity for physiological plasticity in response to DTFs in the tadpoles of species inhabiting environments characterised by different degrees of diurnal temperature variability. The second experimental chapter (Chapter 3) investigates whether exposure to UV influences physiological responses to DTFs. The final experimental chapter (Chapter 4) investigates the interaction between temperature variability and UV exposure on oxidative damage, antioxidant activity, the induction of Hsps and upper thermal limits. Chapters 2 and 3 are published in the *Journal of Experimental Biology* (Kern et al., 2015a; Kern et al., 2014), and Chapter 4 is published in *Comparative Biochemistry and Physiology – Part A* (Kern et al., 2015b). As such, each chapter is written as a complete scientific manuscript with an abstract, introduction, methods, results and discussion. The final chapter of this thesis (Chapter 5) summarises the findings of the experimental chapters, and discusses ideas for future research directions that arose during the completion of this thesis.
PHYSIOLOGICAL RESPONSES OF ECTOTHERMS TO DAILY TEMPERATURE VARIATION

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.

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 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, 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 which reduces 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 plasticity in response to long term temperature changes, 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 gain selective advantage from mechanisms that reduce the thermal sensitivity of physiological traits in response to increased DTFs, in order to reduce the metabolic cost of exposure to high temperatures under such novel conditions (Sinclair et al., 2006; Williams et al., 2012). Ectotherms from environments characterised by large DTFs may 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 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*, *L. 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.

Materials and Methods

Study species

We investigated the response to DTFs in tadpoles of three species that develop in different thermal environments; *L. peronii* (Duméril and Bibron 1841), *L. tasmaniensis* (Günther 1858) and *P. 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 (Figure 2.1). 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 thermal performance curves (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 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 St Lucia, QLD, 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 three locations at each collection site every hour for one month (iButton, Maxim Integrated Products Inc., San Jose, CA, USA).
Figure 2.1. 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.
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 (Figure 2.1). 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 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 (CT<sub>max</sub>), or at one of six temperatures for resting metabolic rate (RMR) or maximum burst swimming performance (U<sub>max</sub>). We then recorded body mass (M<sub>b</sub>, in g), body length and tail length (BL and TL respectively, in mm) and the 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 (U<sub>max</sub>)

U<sub>max</sub> 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<sup>-1</sup> 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 tap water to a depth of 3 cm to prevent vertical movement, and semi-submerged in a water-bath 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 $U_{\text{max}}$.

**Resting metabolic rate (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 water-bath set to the test temperature ($\pm$0.5°C), and after 10 min to allow tadpoles to recover from handling, respirometers were sealed with three-way taps and left for 40–60 min, depending on the test temperature (higher temperatures require less time). The respirometers were fitted with an oxygen-sensitive fluorescent Sensor Spot (PreSens, Regensburg, Germany) and aquatic oxygen partial pressure was determined non-invasively by measuring the fluorescence of the sensor spot through the plastic wall of the respirometer. A fibre-optic cable connected to a Fibox3 reader was used to capture and record fluorescence readings. Continuous, simultaneous temperature recordings of the water-bath allowed for the correction of O$_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:

$$\dot{V}O_2 = (\Delta O_2 \times V)/T$$

Where $\Delta O_2$ 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, lactate dehydrogenase (LDH), and two enzymes involved in aerobic respiration, cytochrome c oxidase (COX) and citrate synthase (CS). We used tail muscle dissected from tadpoles for swimming performance and metabolic rate measurements \((N = 10–12)\). Tissue samples \((0.022–0.053 \text{ g})\) were homogenised in lysis buffer \((1:20 \text{ CS and COX, 1:100 LDH; 50 mmol \text{l}^{-1} \text{ imidazole, 2 mmol \text{l}^{-1} \text{ MgSO}_4, 5 mmol \text{l}^{-1} \text{ EDTA, 0.1\% Triton X-100 and 1 mmol \text{l}^{-1} \text{ glutathione, pH 7.5}})\) 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.

**Critical thermal maximum (CT\text{max})**

CT\text{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 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. CT\text{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\text{max} data were analysed using ANOVA. \(M_b\) was included as a covariate in the analysis of CT\text{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 TPC in response to different thermal environments (Angilletta, 2006; Arrighi et al., 2013). For tadpole RMR and \(U_{\text{max}}\), we used non-linear regression to fit the quadratic function, \(y = ax^2 + bx + c\), to our data to describe TPC through three parameters: \(a\), the
curvature of the apex; \( b \), the slope; and \( c \), the y-intercept. Other nonlinear functions often used for TPCs (i.e. Weibull, Gaussian; Angilletta, 2009) were unable to be fit to our data which failed to meet some requirements of these models. Treatment and test temperature were included as fixed effects in these models as well as \( M_0 \) and TL as covariates for RMR and \( U_{\text{max}} \), respectively.

Enzyme activity was analysed using linear mixed-effects models with residuals weighted using a power variance function to achieve homogeneity of variance. 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.

Results

Water temperatures

The mean water temperature of shallow pools at \( P. \text{ornatum} \) collection sites was 4.7 ± 0.3°C, with average daily fluctuations of 17.5 ± 0.3°C. Deep pools at the geographical location where \( L. \text{tasmaniensis} \) 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 \( L. \text{peronii} \) collection sites had a mean of 27.3±0.1°C and average daily fluctuations of 5.9±0.4°C (Figure 2.1).

Mortality, development time and body condition

No tadpoles of \( L. \text{peronii} \) or \( L. \text{tasmaniensis} \) in the large DTF treatment survived. Mortality of \( L. \text{tasmaniensis} \) tadpoles in the small DTF treatment was significantly higher (58%) than that of tadpoles in the control treatment (39%, \( N = 340, \chi^2 = 12.79, P < 0.001 \)). Mortality of \( L. \text{peronii} \) tadpoles was not different between the small DTF treatment (38%) and the control treatment (29%). Mortality of \( P. \text{ornatum} \) 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, $\chi^2_1 = 135.11$, *P* < 0.001; *L. tasmaniensis*, *N* = 163, $\chi^2_1 = 118.97$, *P* < 0.001). Conversely, development time was shorter for *P. ornatum* tadpoles in both fluctuating treatments compared with the control (*N* = 245, $\chi^2_2 = 32.86$, *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 2.1).

Body mass and BL of *L. peronii* tadpoles was not significantly affected by treatment, whereas TL was significantly reduced by small DTFs (Table 2.1; TL, $F_{1,188} = 8.27$, *P* = 0.005). For both *L. tasmaniensis* and *P. ornatum*, *M_b*, BL and TL were all significantly reduced in tadpoles in small DTFs (Table 2.1; *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).

**Maximum Burst swimming performance (U\(_{\text{max}}\))**

*U\(_{\text{max}}\)* was not significantly affected by treatment in any species (Figure 2.2). 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*; $y = ax^2 + bx + c$, Table 2.2). 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 (Figure 2.3). There was no effect of treatment on RMR of the other species. Test temperature affected RMR through the y-intercept for *L. tasmaniensis* (Figure 2.3) 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 2.1.** 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 (°C)</th>
<th>Development* (days)</th>
<th>Mb (g)</th>
<th>BL (mm)</th>
<th>TL (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. peronii</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>90.79 ± 0.72</td>
<td>338.24 ± 7.41</td>
<td>12.44 ± 0.1</td>
<td>26.20 ± 0.3</td>
</tr>
<tr>
<td>20-30</td>
<td>115.55 ± 0.86</td>
<td>320.95 ± 5.4</td>
<td>12.46 ± 0.1</td>
<td>25.12 ± 0.25</td>
</tr>
<tr>
<td>L. tasmaniensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>156.43 ± 1.1</td>
<td>463.13 ± 8.92</td>
<td>14.16 ± 0.12</td>
<td>28.03 ± 0.24</td>
</tr>
<tr>
<td>20-30</td>
<td>200.93 ± 0.65</td>
<td>370.12 ± 9.24</td>
<td>13.46 ± 0.13</td>
<td>24.1 ± 0.25</td>
</tr>
<tr>
<td>P. ornatum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>29.75 ± 0.38</td>
<td>243.26 ± 6.16</td>
<td>11.48 ± 0.12</td>
<td>19.77 ± 0.21</td>
</tr>
<tr>
<td>20-30</td>
<td>25.60 ± 0.33</td>
<td>182.66 ± 4.62</td>
<td>10.46 ± 0.11</td>
<td>17.78 ± 0.22</td>
</tr>
<tr>
<td>18-38</td>
<td>25.74 ± 0.46</td>
<td>140.45 ± 3.6</td>
<td>9.67 ± 0.09</td>
<td>17.02 ± 0.2</td>
</tr>
</tbody>
</table>

DTF, daily thermal fluctuation; Mb, 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.

**Table 2.2.** ANOVA table from quadratic regression on RMR data.

<table>
<thead>
<tr>
<th></th>
<th>a</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f</td>
<td>F-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Test temperature</td>
<td>1,74</td>
<td>0.93</td>
<td>0.34</td>
</tr>
<tr>
<td>Treatment</td>
<td>1,74</td>
<td>4.85</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Data are for parameters from the function: \( y = ax^2 + bx + c \).
There was a significant effect of treatment and test temperature on RMR in *L. tasmaniensis*. 
Figure 2.2. Maximum burst swimming performance of tadpoles raised in control and fluctuating temperatures. Maximum burst swimming performance ($U_{\text{max}}$, 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.
Figure 2.3. 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).
Metabolic enzyme activity

In *L. peronii* tadpoles, the activity of LDH and CS enzymes was significantly higher in tadpoles from the small DTF treatment than in tadpoles from the control treatment (Figure 4A,B; Appendix 2.1). 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 activity in tadpoles from the control treatment ($F_{1,21} = 6.07$, $P = 0.02$). The activity of COX was significantly affected by test temperature ($F_{1,118} = 401.75$, $P < 0.001$), but was not significantly different between treatments (Figure 2.4C).

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 (Figure 2.4D–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 ($F_{1,22} = 6.72$, $P = 0.02$; Figure 2.4E). 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 (Appendix 2.1).

In *P. ornatum* tadpoles, only the activity of CS was significantly different between treatments (Figure 4H). CS activity was significantly lower in tadpoles from the large DTF treatment compared with that of tadpoles from the control and small DTF treatments, and CS activity in the small DTF treatment was significantly lower than the 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 (Figure 4I; Appendix 2.1). 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 experiencing small DTFs 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.
Figure 2.4. 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 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) treatments, tested at 13, 18, 23, 28, 33 and 38°C.
Tadpoles of all three species in the small DTF treatment had a significantly higher critical thermal maximum ($CT_{\text{max}}$) than tadpoles in the control treatment (Figure 2.5; $L. \text{peronii}$, $F_{1,14} = 18.236$, $P < 0.001$; $L. \text{tasmaniensis}$, $F_{1,15} = 52.52$, $P < 0.001$; $P. \text{ornatum}$, $F_{2,16} = 37.80$, $P < 0.001$). $CT_{\text{max}}$ for $P. \text{ornatum}$ 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 $CT_{\text{max}}$ of tadpoles that experienced small and large DTF. $Platyplectrum \text{ornatum}$ showed a significant interaction between treatment and $M_b$ for this trait ($F_{1,16} = 4.25$, $P = 0.03$).

**Figure 2.5.** Critical thermal maxima ($CT_{\text{max}}$) of tadpoles raised in stable and fluctuating temperatures. $CT_{\text{max}}$ of tadpoles of (A) $L. \text{peronii}$, (B) $L. \text{tasmaniensis}$ and (C) $P. \text{ornatum}$ 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.

**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 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 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 TPC.

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.

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). Plasticity of upper thermal limits was not sufficient for *Limnodynastes* species to overcome lethal consequences of exposure to large DTFs. Both *Limnodynastes* species suffered 100% mortality in the large DTF treatment. This suggests that during embryonic/early larval development the temperatures experienced in this treatment were beyond the upper thermal limits of these species, at least for prolonged exposure, or cumulative exposure to the highest temperatures. These species were unable to overcome the challenges of such large DTFs or exposure to the peak temperatures experienced, although *L. tasmaniensis* is found in habitats which experience this range of temperatures. In the field, microhabitat selection and behavioural thermoregulation may buffer developing tadpoles (although not embryos) from the consequences of temperature fluctuations. Examination of behavioural and physiological flexibility together may reveal more about the capacity to overcome the challenges of temperature extremes.

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; Kjörgaard 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.
Appendix 2.1. 95% confidence intervals of metabolic enzyme activity. Enzyme activities (µmol g⁻¹ min⁻¹) of (A) lactate dehydrogenase (LDH) in *L. peronii*, (B) LDH and cytochrome *c* oxidase (COX) in *L. tasmaniensis* and (C) citrate synthase (CS) and COX in *P. ornatum* tadpoles from the control (24°C; blue), small DTF (20-30°C; red) and large DTF treatments (18-38°C; green).
TEMPERATURE AND UV-B INSENSITIVE PERFORMANCE IN TADPOLES OF THE ORNATE BURROWING FROG: AN EPHEMERAL POND SPECIALIST

Abstract

Animals may overcome the challenges of temperature instability through behavioural and physiological mechanisms in response to short and long term temperature changes. When ectotherms face the challenge of large diurnal temperature fluctuations one strategy may be to reduce the thermal sensitivity of key traits in order to maintain performance across the range of temperatures experienced. Additional stressors may limit the ability of animals to respond to these thermally challenging environments through changes to energy partitioning, or interactive effects. Ornate burrowing frog (*Platyplectrum ornatum*) tadpoles develop in shallow ephemeral pools that experience high diurnal thermal variability (>20°C) and can be exposed to high levels of ultraviolet-B (UV-B) radiation. Here we investigated how development in fluctuating verses stable temperature conditions in the presence of high or low UV-B radiation influences thermal tolerance and thermal sensitivity of performance traits of *P. ornatum* tadpoles. Tadpoles developed in either stable (24°C) or fluctuating temperatures (18-32°C) under high or low UV-B conditions. Tadpoles were tested for upper critical thermal limits and thermal dependence of resting metabolic rate and maximum burst swimming performance. We hypothesised that developmental responses to thermal fluctuations would increase thermal tolerance and reduce thermal dependence of physiological traits, and that trade-offs in the allocation of metabolic resources towards repairing UV-B induced damage may limit the ability to maintain performance over the full range of temperatures experienced. We found that *P. ornatum* tadpoles were thermally insensitive for both burst swimming performance, across the range of temperatures tested, and resting metabolic rate at high temperatures independent of developmental conditions. Maintenance of performance led to trade-offs for growth under fluctuating temperatures and UV-B exposure. Temperature treatment and UV-B exposure had an interactive effect on upper critical thermal limits possibly due to the up-regulation of the cellular stress response. Thermal independence of key traits may allow *P. ornatum* tadpoles to maintaining performance in the thermal variability inherent in their environment.
Introduction

Thermal variability can impact upon the fitness of ectotherms through effects on growth, reproduction and whole animal performance as a consequence of the thermal sensitivity of underlying physiological processes (Huey and Stevenson, 1979; Huey, 1982). Due to the non-linear response of metabolic rate to temperature change, fluctuating temperatures can increase the energy demand required for cell maintenance at higher temperatures. During development, fluctuating temperatures can lead to increased rates of development and a reduction in body size (Dong et al., 2006; Niehaus et al., 2006; Dhillon and Fox, 2007; Du and Shine, 2010) compared to those developing at the equivalent mean temperature (Ruel and Ayres, 1999; Williams et al., 2012). As such, animals may employ behavioural and/or physiological strategies to mitigate the challenges associated with fluctuating environmental temperatures. Thermally variable conditions lead to increased thermal tolerances in some adult aquatic ectotherms (Feldmeth et al., 1974; Widdows, 1976; Sinclair et al., 2006) which suggests that exposure to thermally variable environments can improve performance breadth in some cases. It has yet to be established how variable conditions effect the thermal dependence of performance traits and the physiological mechanisms that underpin them (although see Niehaus et al., 2011). Responses that increase thermal tolerance and reduce the thermal dependence of physiological processes and performance traits may buffer animals from the costs associated with thermally variable environments (Schaefer and Ryan, 2006; Williams et al., 2012) and allow them to remain active across the range of temperatures experienced.

The large diurnal thermal variability characteristic of some aquatic environments (i.e. tidal pools and ephemeral pools) can be due to solar heating of such exposed environments. Animals inhabiting these environments are therefore exposed to high levels of ultraviolet-B (UV-B) radiation. UV-B radiation causes cellular damage due to the absorption of radiation by DNA, which must be repaired to limit disruption to DNA transcription (Friedburg et al., 2006). The consequences of UV-B exposure during development include reduced growth rates (Tevini, 1993; Caldwell et al., 1998), reduced locomotor ability (Álvarez and Nicieza, 2002), reduced metabolic scope (Ylonen et al., 2004) and increased time to metamorphosis in tadpoles (Belden et al., 2000; Pahkala et al., 2001; Belden and Blaustein, 2002; Pahkala et al., 2003). These effects indicate energy allocation to repair UV-B induced DNA damage (Alton et al., 2012). Increased maintenance costs and energy allocation during UV-B exposure may divert energy from compensatory mechanisms to
deal with thermal variability in order to meet energy requirements for growth and development.

Interactive effects of temperature and UV-B radiation may influence the thermal tolerance and thermal sensitivity of performance traits. UV-B radiation interacts with environmental factors such as pH, temperature and predator cues (Pahkala et al., 2002; van Uitregt et al., 2007; Alton et al., 2010) to produce synergistic effects on animal mortality rates. UV-B radiation has been shown to have temperature dependent effects on growth, survival and thermal tolerance (Winckler and Fidhiany, 1996, 1999; van Uitregt et al., 2007), but how these factors interact to influence physiological responses to thermal variability have not been considered. Investigating these types of interactions will broaden our knowledge of the influence of temperature and other environmental factors on the thermal sensitivity of performance traits (Clusella-Trullas et al., 2011).

The challenge of dealing with large diurnal thermal variation and high UV-B radiation is pertinent for the larvae of amphibian species that breed in ephemeral pools of arid and semi-arid zones. Species such as the Australian ornate burrowing frog (*Platyplectrum ornatum*, Gray, 1841) breed after heavy rain in ephemeral water bodies, including small puddles and development is rapid (21 days; Barker et al., 1995) to accommodate a short hydroperiod. Breeding pools are often exposed and can experience considerable diurnal thermal variation (> 20°C; Figure 3.1) and high UV-B radiation (P. Kern obs.) associated with the solar warming of their ephemeral habitats (Blaustein et al., 2001). Furthermore, high metabolic demands for growth during development may limit metabolic scope (Killen et al., 2007; Rombough, 2011), and may reduce the energy available for physiological mechanisms to buffer performance from thermal variability and the effects UV-B in their environments. As such, *P. ornatum* tadpoles provide a good system to investigate interactive effects between thermal variability and UV-B radiation on thermal tolerance and the thermal sensitivity of performance traits, and will allow for a greater understanding of how animals allocate resources during development in these environments.

In this study, we aimed to determine physiological responses of *P. ornatum* tadpoles to the thermal variability and UV-B exposure inherent in their environment. To assess this we examined the interactive influence of UV-B radiation and fluctuating temperatures on thermal tolerance and the thermal sensitivity of performance traits. We examined resting metabolic rate in combination with burst swimming performance, an important predator
avoidance mechanism for tadpoles (Wilson and Franklin, 1999), to determine if key fitness traits were buffered from environmental thermal variability through reduced thermal sensitivity. We hypothesised that developmental responses to thermal fluctuations would reduce thermal dependence of physiological traits, and that interactive effects between fluctuating temperatures and UV-B radiation may reduce the ability of organisms to maintain these performance traits over the full range of temperatures experienced.

Materials and Methods

Egg masses of *P. ornatum* were collected from flooded road sides in known breeding habitats near Dalby, Queensland. Egg masses were kept at ~24°C overnight before being transported to The University of Queensland. Once free swimming (1 day after collection), tadpoles were randomly allocated into 1 L replicate containers (8 per treatment) at an initial density of 30 tadpoles per litre. Replicates were maintained in 35 L water baths to ensure uniform temperature conditions within each treatment. Tadpoles were introduced to temperature treatments in the evening when temperature cycles reached 24°C (Figure 3.2). UV-B exposure commenced the following morning. Tadpoles where fed boiled spinach once a day, with excess food removed and water quality checked daily and changed as needed.
Figure 3.2. Temperature and UV-B treatments. *P. ornatum* tadpoles developed in either fluctuating (18-32°C; solid line) or constant (24°C; dotted line) temperature treatments. Tadpoles in UV+ treatments were exposed to UV-B radiation for 13 hours per day (light grey; 6:00-19:00) with an additional high UV-B peak for 5 hours per day (dark grey; 36±4 µW cm² between 10:00-15:00). Tadpoles in UV- treatments were exposed to this same lighting regime using generic fluorescent bulbs (8.5±1.5 µW cm² between 6:00-19:00). The black and white bar represents photoperiod.

Tadpoles were exposed to a factorial combination of thermal variability and UV-B radiation: stable temperature (24 ± 1°C) or fluctuating temperatures (18-32°C with a mean of 24 ± 1°C) and high (UV+) or low (UV-) UV-B exposure (Figure 3.2). Cycling water temperatures were maintained with aquarium heaters (55 W, Aqua One) submerged in the surrounding water bath, connected to electronic timers. Aquarium water pumps were used to create uniform temperatures across replicate containers. UV-B radiation was generated using 40 W linear fluorescent bulbs (Repti-Glo Exo Tera, Montreal, Canada). Larvae in the UV+ treatments were exposed to 4.7 ± 1.5 µW cm⁻² of UV-B for 13 h per day with an additional peak of 36 ± 4 µW cm⁻² for five hours occurring in the middle of the day. Replicating ambient UV-B levels has been shown to be lethal for the tadpoles of some species (van Uitregt et al., 2007), and so UV-B levels were ~6 % of ambient radiation to enable us to investigate non-lethal effects (Alton et al., 2010; Alton et al., 2012) while maintaining sufficient survival rates (van Uitregt et al., 2007; Alton et al., 2011). Tadpoles in the UV- treatments were exposed to the same lighting regime generated by generic
linear fluorescent bulbs (UV-B 8.5 ± 1.5 µW cm⁻²). The cumulative total radiation (UV-B) received over 24 h was 7817 and 3950 J m⁻² for UV+ and UV- treatments respectively. In summary, tadpoles were exposed to one of four treatment groups: 24°C/UV+, 24°C/UV-, 18-32°C/UV+ and 18-32°C/UV-.

We recorded survival to stage 35-37 (Gosner, 1960) and the time taken to reach this stage. Stage 35-37 is a relatively stable time in development (Gosner, 1960) before hind limbs are large enough to effect swimming movement (Hoff and Wassersug, 2000). At this developmental stage, tadpoles were tested for either critical thermal maximum (CT_max), or at one of five temperatures for resting metabolic rate (RMR) or maximum burst swimming performance. We then recorded body mass (M_b, in g) and body (BL) and tail length (mm). We randomly selected one tadpole from each replicate container to be assessed for each performance measure so that each replicate had a tadpole included in each test at each temperature. After testing, tadpoles were euthanased by exposure to Aquis-S (New Zealand LTD).

**Critical thermal maximum (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 per minute in a water bath (24-43.7°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 measures were non-fatal and all tadpoles recovered.

**Resting metabolic rate (RMR)**

Resting metabolic rate was calculated from oxygen consumption using closed system respirometry (Sinclair et al., 2006) at five test temperatures (18, 23, 28, 33, and 38°C) to generate a thermal performance curve. 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. 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 minutes to allow tadpoles to recover from handling, respirometers were sealed with three way taps and left for 40–90
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 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 rate ($\dot{V}O_2$: mL O2 h\(^{-1}\)) was calculated using the following formula:

$$\dot{V}O_2 = \frac{(\Delta O_2 \times V)}{T}$$

Where $\Delta O_2$ is the change in oxygen in the chamber (mL O2 L\(^{-1}\)), V is the volume of the respirometer container (L) and T is time (min).

**Burst swimming performance ($U_{max}$)**

Burst swimming performance was assessed at five temperatures (18, 23, 28, 33, and 38°C) to generate a thermal performance curve. As for RMR, tadpoles were brought to each test temperature slowly to prevent thermal shock. 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 3cm 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 recorded using a high-speed digital camera (Canon EX-FH25) recording at 240Hz, pointed at a mirror positioned at a 45 degree angle above the burst arena. The first 100 ms following the completion of the C-start were analysed (Tracker Video Analysis and Modelling Tool, Open Source Physics) 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 ($U_{max}$).
Statistical Analysis

The thermal sensitivity of activity rates for RMR and burst swimming performance were calculated as $Q_{10} = (R_2/R_1)^{(10/T_2-T_1)}$, where R represents the rate at temperature (T) 1 and 2. Thermal sensitivities were calculated for the entire range of test temperatures, as well as for the upper (28-38°C) and lower (18-28°C) temperature ranges.

Morphometric, survival and $CT_{\text{max}}$ data were analysed using generalised linear models including temperature, UV-B treatment and an interaction term in the maximal model. For RMR and burst swimming performance non-linear regression was used to fit data to a quadratic function to describe the thermal performance curves. Test temperature, temperature treatment, UV-B treatment and an interaction term were included in the maximal modal as well as body mass and body length respectively as co-variates. All analyses were done using the R statistical software package (R Development Core Team, 2014). Where indicated, Bonferroni’s post-hoc pairwise analyses were used. Data are presented as mean ± s.e.m.

Results

Survival, development time and body condition

For survival, there was a significant interaction between treatment temperature and UV-B treatment ($F_{1,28} = 5.12$, $P = 0.03$). Survival was unaffected by high (UV+) or low (UV−) UV-B for tadpoles in the 24°C treatment ($24^\circ\text{C/UV+} = 41.3 \pm 2.72.5\%$; $24^\circ\text{C/UV−} = 38.8 \pm 2.2\%$), whereas for tadpoles in the 18–32°C temperature treatment, high UV-B reduced survival ($18–32^\circ\text{C/UV+} = 35.0 \pm 4.6\%$; $18–32^\circ\text{C/UV−} = 46.7 \pm 2.4\%$). The average time for tadpoles to reach development stage 35–37 (Gosner, 1960) was 39±1 days with no difference in development time between tadpoles from temperature ($F_{1,374} = 0.44$, $P = 0.51$) and UV-B treatments ($F_{1,373} = 0.37$, $P = 0.36$). At stage 35–37, body mass and body and tail length were significantly different between treatments. The body mass of tadpoles was significantly affected by both temperature treatment ($F_{1,374} = 22.25$, $P < 0.001$) and UV-B treatment ($F_{1,373} = 4.08$, $P = 0.04$; Figure 3.2) independently, with no interactive effect ($F_{1,372} = 0.61$, $P = 0.61$).
Fluctuating temperatures reduced body mass and further reductions were seen in UV-B exposed tadpoles. Temperature treatment had the greatest influence on body mass with tadpoles from the thermally stable treatments (24°C/UV+, $N = 94$, $M_b = 0.27 \pm 0.01$ g; 24°C/UV–, $N = 91$, $M_b = 0.28 \pm 0.01$ g) being heavier than tadpoles from thermally fluctuating treatments (18–32°C/UV+, $N = 84$, $M_b = 0.24 \pm 0.01$ g; 18–32°C/UV–, $N = 107$, $M_b = 0.25 \pm 0.01$ g). Within temperature treatments, the exposure to high UV-B reduced body mass, with tadpoles in the UV+ treatment being significantly lighter than those in the UV– treatment within the same temperature regime. Temperature treatment also significantly affected body length ($F_{1,368} = 15.35$, $P < 0.001$; 24°C/UV+, $N = 91$, $BL = 12.18 \pm 0.09$ mm; 24°C/UV–, $N = 90$, $BL = 12.21 \pm 0.09$ mm; 18–32°C/UV+, $N = 83$, $BL = 11.82 \pm 0.10$ mm; 18–32°C/UV–, $N = 106$, $BL = 11.74 \pm 0.10$ mm), tail length ($F_{1,368} = 7.77$, $P = 0.006$; 24°C/UV+, tail length = 16.10 \pm 0.14 mm; 24°C/UV–, tail length = 16.0 \pm 0.13 mm; 18–32°C/UV+, tail length = 16.05 \pm 0.14 mm; 18–32°C/UV–, tail length = 16.69 \pm 0.17 mm) and total length ($F_{1,368} = 14.11$, $P < 0.001$; 24°C/UV+, $TL = 28.0 \pm 0.21$ mm; 24°C/UV–, $TL = 27.74 \pm 0.19$ mm; 18–32°C/UV+, $TL = 28.38 \pm 0.21$ mm; 18–32°C/UV–, $TL = 28.9 \pm 0.23$ mm) of tadpoles, with tadpoles in 18–32°C treatments being shorter in each measure than tadpoles that developed at a constant 24°C. UV-B treatment had no effect on body length parameters ($BL$, $F_{1,367} = 0.25$, $P = 0.62$; tail length, $F_{1,367} = 1.35$, $P = 0.25$; $TL$, $F_{1,367} = 0.36$, $P = 0.55$).

![Figure 3.3](image-url) **Figure 3.3.** The effects of fluctuating temperatures and UV-B radiation on body mass (g) of *P. ornatum* tadpoles. Both fluctuating temperatures ($F_{1,374} = 22.25$, $P < 0.001$) and the presence of UV-B ($F_{1,373} = 4.08$, $P = 0.04$) significantly reduced body mass. 24°C/UV+ $N = 94$, 24°C/UV− $N = 91$, 18–32°C/UV+ $N = 84$, 18–32°C/UV+ $N = 107$. Letters denote significant differences.
There was a significant interaction ($N = 8$, $F_{1,28} = 22.91$, $P < 0.001$) between treatment temperature and UV-B treatment which influenced $CT_{\text{max}}$ (Figure 3.4). The $CT_{\text{max}}$ of tadpoles from both thermal treatments was not significantly different when exposed to low levels of UV-B (UV-). Exposure to high levels of UV-B (UV+) caused a 0.84°C reduction in $CT_{\text{max}}$ for tadpoles in the stable thermal treatment and a 0.76°C increase in $CT_{\text{max}}$ for tadpoles in the fluctuating thermal treatment.

![Graph showing CT_max vs Temperature treatment]

**Figure 3.4.** The interactive effects of temperature treatment and UV-B radiation on $CT_{\text{max}}$ of tadpoles of *P. ornatum*. There was a significant interaction between temperature treatment and UV-B treatment ($F_{1,28} = 22.91$, $P < 0.001$) for $CT_{\text{max}}$. $N = 8$ for all treatments. Letters denote significant differences.

**Resting metabolic rate (RMR)**

There was no effect of rearing temperature or UV-B treatment on RMR (Figure 3.5, Table 3.1). Test temperature itself significantly affected oxygen consumption ($N = 7-11$ for all test temperatures and treatments, $F_{1,167} = 306.13$, $P < 0.001$) with oxygen consumption generally increasing with temperature, although oxygen consumption at 38°C was not significantly different from oxygen consumption at 28 or 33°C (Bonf. adj. $P > 0.05$). The $Q_{10}$ for RMR showed reduced thermal sensitivity at the higher temperatures where $Q_{10}(28-38°C)$ ranged between 1.1-1.3 compared to lower temperatures where $Q_{10}(18-28°C)$ ranged from 2.0-2.4 (Table 3.2).
Figure 3.5. The thermal dependence curves of RMR for *P. ornatum* tadpoles in thermally stable or thermally variable treatments and with or without UV-B radiation. Oxygen consumption was not significantly different between tadpoles from the fluctuating temperature treatment compared to those in stable temperatures. UV-B exposure had no effect on oxygen consumption. Oxygen consumption increased with test temperature for the lower range of temperatures tested ($F_{1,167} = 306.13$, $P < 0.001$).

Table 3.1. ANOVA table from non-linear regression on RMR data

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<tr>
<td>Temperature</td>
<td>1,157</td>
<td>0</td>
<td>0.99</td>
<td>1,157</td>
<td>1.03</td>
<td>0.31</td>
<td>1,157</td>
<td>0.01</td>
<td>0.92</td>
</tr>
<tr>
<td>UV</td>
<td>1,157</td>
<td>0.326</td>
<td>0.57</td>
<td>1,157</td>
<td>1.17</td>
<td>0.28</td>
<td>1,157</td>
<td>0.90</td>
<td>0.35</td>
</tr>
<tr>
<td>Body Mass</td>
<td>1,157</td>
<td>285.41</td>
<td>&lt;.0001</td>
<td>1,157</td>
<td>0.10</td>
<td>0.76</td>
<td>1,157</td>
<td>0.73</td>
<td>0.39</td>
</tr>
<tr>
<td>Temperature X</td>
<td>1,157</td>
<td>0.37</td>
<td>0.55</td>
<td>1,157</td>
<td>0.80</td>
<td>0.37</td>
<td>1,157</td>
<td>0.11</td>
<td>0.74</td>
</tr>
</tbody>
</table>

RMR, resting metabolic rate.
Data are for parameters from the function: $y = ax^2 + bx + c$.

Table 3.2. Q10 values for RMR for *P. ornatum* tadpoles in thermally stable or thermally variable treatments and with or without UV-B radiation.

<table>
<thead>
<tr>
<th></th>
<th>18-32°C/UV-</th>
<th>18-32°C/UV+</th>
<th>24°C/UV-</th>
<th>24°C/UV+</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_{10}(18-38)$</td>
<td>1.55</td>
<td>1.55</td>
<td>1.76</td>
<td>1.62</td>
</tr>
<tr>
<td>$Q_{10}(18-28)$</td>
<td>1.95</td>
<td>2.12</td>
<td>2.61</td>
<td>2.36</td>
</tr>
<tr>
<td>$Q_{10}(28-38)$</td>
<td>1.24</td>
<td>1.13</td>
<td>1.18</td>
<td>1.11</td>
</tr>
</tbody>
</table>
Burst swimming performance

There was no effect of rearing temperature or UV-B treatment on maximum burst swimming performance (Figure 3.6, Table 3.3). Test temperature significantly affected burst swim speed ($N = 7-12$ for all treatments and test temperatures, $F_{1,160} = 7.72, P = 0.006$) although this difference only lay between 18 and 33°C test temperatures (Bonf. adj. $P = 0.009$). Burst swimming performance at all other test temperatures were not significantly different. $Q_{10}(18-38°C)$ values for maximum burst swimming performance ranged between 1.1-1.2 over the entire range of temperatures indicating low thermal sensitivity (Table 3.4).

![Graph](image)

**Figure 3.6.** The interactive effects of thermal variability and UV-B radiation on the thermal dependence of burst swimming speed in *P. ornatum* tadpoles. Swimming performance was not significantly affected by temperature or UV-B treatment. Swimming performance was found to be thermally insensitive, with burst swimming performance only significantly different ($F_{1,160} = 7.72, P = 0.006$) between 18 and 33°C (Bonf. adj. $P = 0.009$).
Table 3.3. ANOVA table from non-linear regression on maximum burst swimming data

<table>
<thead>
<tr>
<th></th>
<th>a</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f</td>
<td>F-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Temperature</td>
<td>1,150</td>
<td>3.69</td>
<td>0.06</td>
</tr>
<tr>
<td>UV</td>
<td>1,150</td>
<td>0.58</td>
<td>0.45</td>
</tr>
<tr>
<td>Tail Length</td>
<td>1,150</td>
<td>6.44</td>
<td>0.01</td>
</tr>
<tr>
<td>Temperature X UV</td>
<td>1,150</td>
<td>2.78</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Data are for parameters from the function: \( y = ax^2 + bx + c \).

Table 3.4. \( Q_{10} \) values for maximum burst swimming performance for \( P. ornatum \) tadpoles in thermally stable or thermally variable treatments and with or without UV-B radiation.

<table>
<thead>
<tr>
<th></th>
<th>18-32°C/UV-</th>
<th>18-32°C/UV+</th>
<th>24°C/UV-</th>
<th>24°C/UV+</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Q_{10}(18-38) )</td>
<td>1.17</td>
<td>1.06</td>
<td>1.07</td>
<td>1.08</td>
</tr>
<tr>
<td>( Q_{10}(18-28) )</td>
<td>1.40</td>
<td>1.30</td>
<td>0.98</td>
<td>0.88</td>
</tr>
<tr>
<td>( Q_{10}(28-38) )</td>
<td>0.98</td>
<td>0.86</td>
<td>1.18</td>
<td>1.32</td>
</tr>
</tbody>
</table>

Discussion

Ornate burrowing frog tadpoles demonstrated innate thermal insensitivity of burst swimming performance and metabolism (at high temperatures) and the allocation of resources to maintain key traits, which reflect the thermal variability of their thermal environment. We predicted that tadpoles would increase thermal tolerance and reduce the thermal dependence of RMR and burst swimming performance in response to fluctuating temperatures, and that interaction with UV-B radiation may reduce this response to fluctuating temperatures. This was not observed, and rather, tadpoles maintained performance independently of environmental temperature and UV-B treatment. Our results suggest that: 1. thermal dependence of RMR and burst swimming performance was unaffected by thermal fluctuations or UV-B exposure in tadpoles of \( P. ornatum \), and 2. ornate burrowing frog tadpoles appear to have innate thermal insensitivity which may allow it to maintain burst swimming performance and physiological function in a thermally challenging environment.
Metabolism which underlies important fitness traits by providing energy for growth, development and performance (Angilletta et al., 2006; Wilson et al., 2007; Seebacher, 2009), is a thermally dependent process. In the absence of mechanisms to reduce the thermal sensitivity of metabolism, fitness may be reduced as a result of variable energy availability. Tadpoles reared in thermally variable conditions retain the same thermal sensitivity of metabolic rate as animals reared in thermally stable conditions. All tadpoles showed reduced thermal sensitivity of metabolism between 28-38°C. While tadpoles cannot avoid the direct effect of temperature on metabolism across the range of temperatures experienced, reduced thermal sensitivity at higher temperatures may buffer tadpoles from high metabolic demands during peak environmental temperatures.

Although metabolic rate was not different between any treatment, temperature effects on metabolism during the warm part of thermal oscillations may disproportionally increase metabolic demand beyond what would be predicted by the mean temperature (Williams et al., 2012). Both temperature and UV-B exposure affected the body condition of tadpoles. For tadpoles in the fluctuating temperature treatment, it appears that high temperatures created a mismatch between metabolic demand and available energy resulting in a reduction of body mass and length. Exposure to high UV-B exaggerated the reduction in body size, above and beyond the effects of reduced density due to high mortality for tadpoles exposed to fluctuating temperatures and high UV-B levels.

Burst swimming performance was almost entirely thermally insensitive suggesting inherent temperature independence for this trait. Burst swimming performance is a key fitness trait for tadpoles which depend extensively upon it for surviving predation and for foraging (Watkins, 1996; Wilson and Franklin, 1999). Ornate burrowing tadpoles appear to overcome temperature effects on burst swimming performance which may allow them to maintain predator avoidance mechanisms in environments characterised by thermal variability. Compared with related anuran species, ornate burrowing frog tadpoles had far lower Q_{10} values for burst swimming performance (Wilson and Franklin, 1999; Niehaus et al., 2011). Our results indicated that *P. ornatum* tadpoles are able to buffer burst swimming performance from the effects of thermal perturbation and that this response occurs independently of developmental conditions. Maintenance of burst swimming performance across a range of temperatures reflects the inherent thermal variability of their developmental environment.
Some killifish also employ this strategy to deal with a similar range of environmental temperature (Fangue et al., 2008). However, metabolic rate in killifish reflects the thermal independence of their swimming performance which allows energy requirements for maintaining constant performance to be met. For *P. ornatum* tadpoles, varying energy availability due to thermodynamic effects on metabolism imposed no constraints on performance over the temperature range tested. The energy required to maintain performance, especially at low temperatures, must represent a large portion of the energy budget of these tadpoles during development, and may reduce energy available for other traits such as growth.

Partitioning of energy from growth to key traits may improve chances of survival. Such compensatory partitioning occurs when stressors impose additional metabolic costs and the energy required to support one trait is supplied by reducing resources available for some other function (Wieser, 1989; Rombough, 2011). Unexpectedly, development time was not influenced by developmental conditions. Fluctuating temperatures have led to shorter larval periods in other amphibians (Niehaus et al., 2006; Měráková and Gvoždík, 2009) and other groups of animals (Shine et al., 1997; Du and Shine, 2010). For tadpoles, increasing development rates allow individuals to escape deteriorating conditions. Our results indicate that although resources are diverted from growth to deal with imposed conditions, the development time of this species was maintained. Perhaps the short development time already represents the minimum possible for this species, which allows them to reproduce in highly temporal breeding habitats. This species appears to allocate resources to key survival traits which allow it to survive in a metabolically costly larval environment.

Surprisingly, temperature treatment alone did not influence the upper thermal limit of tadpoles. This contrasts with the results of numerous studies where thermal fluctuations increased upper thermal limits (Feldmeth et al., 1974; Otto, 1974; Schaefer and Ryan, 2006) close to what would be expected with acclimation to the peak temperature of the fluctuation (Otto, 1974). Extension of thermal tolerance breadth may only occur when the peak of the oscillation is above the thermal optimum of the species (Woiwode and Adelman, 1992), as the upper temperature experienced is likely to be the cue for extending CT_{max} (Heath, 1963). In this study, the temperature at which performance was optimised was approximately 33°C (Figure 3.6), which lies just above the upper peak of the temperature oscillations of the fluctuating treatment group. Therefore, the upper
temperature of the fluctuating temperature treatment may not have been high enough to induce an increase in thermal tolerance.

UV-B exposure had variable effects on \( CT_{max} \) depending on temperature treatment. Exposure to high UV-B radiation resulted in an increase in \( CT_{max} \) for tadpoles in the fluctuating temperature treatment, and a decrease in \( CT_{max} \) for tadpoles held at stable temperatures. Exposure to high levels of UV-B radiation resulted in reduced body mass of tadpoles. Alton et al. (2012) showed that UV-B exposure is energetically costly for tadpoles. The reduced body mass of tadpoles exposed to UV-B may therefore be indicative of these costs with a reduction in energy allocated to growth. Repair of UV-B induced damage and a likely up-regulation of the cell stress response (CSR) may account for some of the costs of UV-B exposure. The CSR plays an important role in preventing and repairing macromolecular damage involving heat shock proteins (Kultz, 2003, 2005). There are strong indications that the induction of heat shock proteins plays a direct or indirect role in determining thermal tolerance (i.e. \( CT_{max} \), Tomanek, 2008; Fangue et al., 2011). Pyrimidine dimers that form when UV radiation damages DNA act as a signal to induce the CSR (Mitchel and Morrison, 1984; Anderson et al., 1988). Short UV wavelengths have been shown to induce heat shock proteins (Mitchel and Morrison, 1984; Anderson et al., 1988). It is possible that tadpoles exposed to UV-B in the fluctuating thermal treatment may have incidentally increased \( CT_{max} \) through induction of the CSR as a reaction to UV-B induced damage. The mechanisms that lead to a reduction in \( CT_{max} \) for tadpoles in constant temperature was not established although previously recognised interactive effects of UV-B radiation and temperature (van Uitregt et al., 2007) may be important in explaining the reduction of thermal tolerance. This interactive effect of temperature and UV-B radiation on upper thermal limits may have important implications for survival in a warming climate (Duarte et al., 2012).

Evidently, for this species, some physiological processes are buffered from environmental variations during development, as the same phenotype for these traits was produced regardless of developmental conditions. This environmental canalisation, which refers to the insensitivity of a trait to environmental factors (Waddington, 1942; Wagner et al., 1997; Debat and David, 2001), protects these traits against environmental perturbation. Canalisation of traits is thought to occur under natural selection and indicates the importance of such traits to fitness as they are maintained independently of environmental conditions (Waddington, 1942; Stearns and Kawecki, 1994).
Our results suggest that canalisation of traits may buffer development from the unpredictable environmental conditions that *P. ornatum* tadpoles experience *in situ*. This species shows inherent capacities to deal with some challenges of diurnal thermal variation through the thermal independence of burst swimming performance and metabolism at high temperatures, the allocation of resources to maintain key traits and a rapid development time. These traits reflect ecological conditions and may allow ornate burrowing frog tadpoles to maintain performance and physiological function over the range of environmental temperatures they experience. While thermal independence of key traits makes these tadpoles robust in dealing with some challenges of thermal variability inherent in their ephemeral environments, canalisation of traits may reduce their ability to respond to increased variability predicted from current climate models. Increased temperatures and temperature variability may cause ephemeral breeding pools to evaporate more quickly. If tadpoles do not have the capacity to increase development rate they may not be able to reach metamorphosis before habitats dry. Furthermore, failure to mitigate energetic costs of fluctuating temperatures may push metabolic demands beyond the capacity of individuals.
CHAPTER 4

PLASTICITY OF PROTECTIVE MECHANISMS ONLY PARTIALLY EXPLAINS INTERACTIVE EFFECTS OF TEMPERATURE AND UVR ON UPPER THERMAL LIMITS

Abstract

Temperature and ultraviolet radiation (UVR) are key environmental drivers that are linked in their effects on cellular damage. Exposure to both high temperatures and UVR can cause cellular damage that result in the up-regulation of common protective mechanisms, such as antioxidant activity and induction of heat shock proteins (Hsps). As such, the interactive effects of these stressors at the cellular level may determine physiological limits, such as thermal tolerance. Furthermore, antioxidant activity is often thermally sensitive, which may lead to temperature dependent effects of UVR exposure. Here we examined the interactive effects of temperature and UVR on upper thermal limits, Hsp70 abundance, oxidative damage and antioxidant (catalase) activity. We exposed tadpoles of striped marsh frogs, *Limnodynastes peronii* to one of three temperature treatments (constant 18°C, constant 28°C and daily fluctuations between 18-28°C) in the presence or absence of UVR. Tadpoles were tested for upper thermal limits (CT$_{\text{max}}$), abundance of Hsp70, oxidative damage and catalase activity. Our results show that upper thermal limits were influenced by an interactive effect between temperature and UVR treatment. For tadpoles kept in cold temperatures, exposure to UVR led to cross-tolerance to high temperatures, increasing CT$_{\text{max}}$. Plasticity in this trait was not fully explained by changes in the lower level mechanistic traits examined. These results highlight the difficulty in predicting the mechanistic basis for the interactive effects of multiple stressors on whole animal traits. Multifactorial studies may therefore be required to understand how complex mechanistic processes shape physiological tolerances, and determine responses to environmental variation.

Introduction

The range of environmental conditions animals can tolerate is associated with the physiological capacity to prevent or repair damage caused by environmental stress (Lindquist and Craig, 1988). These physiological tolerance limits are likely determined by
the collective action of a range of cellular mechanisms that prevent cellular damage. Plasticity in these mechanisms is important for overcoming the potentially negative effects of environmental variation, and is therefore involved in determining species persistence across environments (Calosi et al., 2008; Simon et al., 2015; Stillman, 2003). Thermal tolerance refers to the range of temperatures animals can endure. Plasticity in upper and lower thermal limits in response to temperature change increases stress resistance. For example, acclimation to high temperatures can increase upper thermal limits (Ravaux et al., 2012; Schaefer and Ryan, 2006). Exposure to other abiotic/biotic factors, such as environmental contamination or predators (Sørensen et al., 2011; Pandolfo et al., 2010), can also alter physiological capacities to withstand high temperatures (Jirsa et al., 2013; Healy and Schulte, 2012; Sørensen et al., 2011). However it is the interactive effects of multiple factors that will likely determine thermal tolerances. For example, exposure to daily temperature fluctuations increased upper thermal limits only in the presence of ultraviolet radiation (UVR) resulting in an interactive effect between temperature and UVR on this trait (Kern et al., 2014). As such, understanding the interactive effects of multiple environmental factors on physiological processes will be important in determining what shapes thermal tolerance.

The interactive effects of temperature and UVR on upper thermal limits may be determined by the way these stressors influence important cellular processes. Thermal stress and exposure to UVR can cause cellular damage through protein denaturation (Lindquist and Craig, 1988) and breaking of important structural and chemical bonds (Tevini, 1993) respectively. These stressors are linked in their effects on cellular function as the damage caused by temperature stress and UVR activate common protective mechanisms that maintain the integrity of cellular components during environmental stress (Bonaventura et al., 2006; Flanagan et al., 1995; Kwon et al., 2002). Heat stress can reduce the damage caused by subsequent exposure to UVR (Trautinger et al., 1996), providing evidence for a common protective mechanism against temperature and UVR stress. Such cross-tolerance results when exposure to one stressor increases tolerance to a second stressor (MacMillan et al., 2009) and may help explain why the outcome of multiple stressor studies cannot be predicted from the single stressor studies.

Upper thermal limits are often correlated with the expression of heat shock proteins (Hsps) (Gong and Golic, 2006; Kalosaka et al., 2009). Hsps function as molecular chaperones, which prevent damage when cells experience environmental stress by refolding and
precluding the aggregation of non-native proteins (Feder and Hofmann, 1999b). Hsps prevent damage during both temperature stress and exposure to UVR (Bonaventura et al., 2005; Kwon et al., 2002; Parsell and Lindquist, 1993). Organisms have both constitutively expressed and stress-induced Hsps and plasticity in the abundance and/or induction temperature of both forms are important mechanism associated with flexibility in upper thermal limits (Fangue et al., 2011; Gong and Golic, 2006; Kwon et al., 2002; Lindquist and Craig, 1988; Nakano and Iwama, 2002). The increase in upper thermal limits caused by an interaction between temperature and UVR (Kern et al. 2014) may be explained by cross-tolerance resulting from the expression of Hsps in response to UVR, which subsequently increased tolerance to high temperatures. Predicting how temperature and UVR will shape thermal tolerances is complicated by the potential for such complex responses at the cellular level.

High temperatures and UVR exposure can also cause oxidative damage due to the formation of reactive oxygen species (ROS; Abele et al., 2002; Shick et al., 1996). ROS are produced through oxidative metabolism and are involved in cell signaling. ROS are generated at a greater rate at high temperatures due to thermodynamic effects on metabolism. Additionally, absorption of UVR increases production of ROS (Shiu and Lee, 2005; Shick et al., 1996). Excess ROS are scavenged by antioxidants, but when the production of ROS overwhelms these defences, oxidative stress damages DNA, proteins and lipids (Turrens, 2003). The activities of enzymatic antioxidants such as superoxide dismutase and catalase are temperature sensitive (Ghanizadeh Kazerouni et al., 2015; Meng et al., 2014), and such thermodynamic effects can lead to greater UVR induced oxidative damage at low temperatures (Kong et al., 2012). However, antioxidant activity can thermally acclimate (Meng et al., 2014; Selman et al., 2000; Vinagre et al., 2012), and increase activity after exposure to UVR (Cruces et al., 2013; Han et al., 2009; Yu et al., 2009). Furthermore, high temperatures may both alleviate some harmful effects of exposure to UVR, due to an increase in antioxidant capacity (Han et al., 2009), and cause detrimental effects by preventing the UVR induced increase in antioxidant activity (Cruces et al., 2013). As temperature and UVR can influence ROS production, antioxidant activity and the expression of Hsps, simultaneous exposure to these stressors may result in complex responses within and between cellular response pathways that result in interactive effects on whole-animal level traits. In this paper we test the hypotheses that high temperature and UVR interact to increase upper thermal limits and abundance of Hsp70, and that UVR induced oxidative damage will be greater at cold temperatures due
to thermodynamic effects on antioxidant activity. Determining the interactive effects of multiple variables is essential to understand how complex environmental variation shapes physiological tolerances.

Shallow aquatic habitats can be highly variable environments characterised by thermal variation, periodic high UVR levels, changing oxygen availability, and conspecific competition as pond conditions change (Blaustein et al., 2001; Scholnick, 1994). Ectotherms inhabiting these environments provide a good model to investigate physiological mechanisms underlying environmental tolerance. In this study we investigated the interactive effects of temperature and UVR on upper thermal limits, Hsp70 abundance, oxidative damage and catalase activity in *Limnodynastes peronii* tadpoles. This species is an excellent model to investigate the interactive effects of temperature and exposure to UVR, as it is susceptible to damage from UVR (Alton et al., 2012; Alton et al., 2010), has the capacity to thermally acclimate (Seebacher and Grigaltchik, 2014; Wilson and Franklin, 1999) and shows temperature dependent effects of UVR (van Uitregt et al., 2007).

**Materials and methods**

*Animal collection and maintenance*

Five egg masses of *L. peronii* were collected from water bodies in Brisbane, QLD, Australia (27°30' S, 152°59' E) over two days. Eggs were immediately transported to The University of Queensland where eggs and subsequent tadpoles were maintained at 23°C for one month. After this period equal numbers of tadpoles from each clutch were randomly divided into 1 L replicate tanks (6 per treatment; 36 tanks) with an initial density of 23 tadpoles/container. For each experimental measure, tadpoles were sampled across replicate containers to avoid tank effects. Replicate tanks were maintained in 35 L water baths with aquarium water pumps used to create uniform temperatures across replicates within temperature treatments. Tadpoles were fed boiled spinach daily and water was changed as required. Tadpoles were maintained in 14h: 10 h (light: dark).
**Experimental conditions**

Tadpoles were exposed to one of three temperature treatments: cold (18°C), hot (28°C) or a daily fluctuating treatment (18-28°C daily with a mean of 23°C) in the presence and absence of UVR (UV+/ UV-). Cycling temperatures in the fluctuating treatment were maintained with aquarium heaters (55 W, Aqua One, Southampton, UK) connected to electronic timers set to turn on at 05:00 and off at 15:00. UVR radiation was generated using four 40 W linear fluorescent bulbs (Repti-Glo Exo Tera, Montreal, Canada) suspended above replicate containers. The spectral irradiance (W m^{-2} nm^{-1}) produced in UV+/UV- treatments was measured using a cosine corrector (CC-3-UV-S, Ocean Optics, Dunedin, Florida, USA) and UV-VIS fibre optic cable (400 µm Premium Fiber, Ocean Optics, Dunedin, Florida, USA) connected to a spectrometer (USB2000+ Miniature Fiber Optic Spectrometer, Ocean Optics, Dunedin, Florida, USA) at 8 fixed positions set at the water level of replicate containers in each temperature treatment. We calculated the absolute irradiance (µW cm^{-2}) of UV-B and UV-A at each of the 8 fixed positions (for each temperature treatment) by integrating the spectral irradiance data between 300 – 320 nm, and 320 – 400 nm, respectively. Tadpoles in UV+ treatments were exposed to UVR for 4 h per day (UV-B 25 µW cm^{-2}, UV-A 80 µW cm^{-2}). Ambient levels of UVR at mid-day during the breeding season of *L. peronii* in Brisbane QLD, Australia have been reported as ~500 µW cm^{-2} (van Uitregt et al., 2007). UVR levels were set low enough to enable us to investigate non-lethal effects through maintaining high survival rates (Alton and Franklin, 2012; Alton et al., 2012; Alton et al., 2011). Tadpoles in UV- treatments were maintained under the same lighting conditions but containers were covered with UV retardant film (Handi Homes, Thomastown, Australia) and received negligible amounts of UVR (UV-B 1.6 µ Wcm^{-2}, UV-A 12.5 µW cm^{-2}). Tadpoles were maintained in experimental treatments for four weeks after which mortality was recorded and tadpoles were either tested for critical thermal maximum (CT_{max}), used in a heat shock response protocol for determination of abundance of Hsp70, or euthanased (Benzocaine 250µg/mL Sigma-Aldrich, Castle Hill, Australia) and snap frozen in liquid nitrogen for determination of oxidative damage and antioxidant activity.

**Critical thermal maximum (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 (Duarte et al., 2012; Lutterschmidt and Hutchinson, 1997). Briefly, tadpoles were transferred to a water
bath (N = 12 per treatment) at a common temperature of 23°C and allowed to adjust for one hour. Tadpoles were then exposed to a constant heating rate of 0.5°C per minute 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 measures were non-lethal and all tadpoles recovered. Tadpoles were then weighed and euthanised.

Heat shock exposure

Tadpoles (N = 12 per treatment) were transferred to one of four heat shock temperatures (28, 31, 34 or 36°C) for two hours. Tadpoles were then returned to their treatment temperature (18 or 28°C) to recover for 1 hour. Tadpoles from the 18-28°C treatment were also returned to 28°C. Tadpoles were then euthanased and snap frozen in liquid nitrogen for use in Hsp70 assays.

Determination of Hsp70 abundance

Snap frozen whole tadpoles was homogenised in buffer (1:10 w/v; 1xPBS, 0.5% Triton-X 100, cOmplete, Mini Protease Inhibitor Tablet; Roche, Dee Why, Australia) and centrifuged at 4°C for 10 min at 10000 g. After collecting the resulting supernatant, protein content was determined using a Coomassie Plus (Bradford) Assay (Thermo Scientific, Rockford, USA). Relative protein abundance of constituent (Hsp73) and stress-induced Hsp70 (Hsp72) were determined using gel electrophoresis and Western blotting.

Individual tadpole samples (N = 6/7) were denatured at 70°C for 10 min, and equal amounts of protein (20 µg) were loaded into a 4 – 12 % Bolt BIS-TRIS Plus gel (Life Technologies, Mulgrave, VIC, Australia) and run for 25 min at 200 V. Following electrophoresis proteins were transferred to a Whatman® Westran® PVDF membrane (GE Healthcare, Parramatta, Australia) for 45 min at 60 V. Before beginning, transfer conditions were optimised to ensure complete transfer of proteins. Membranes were blocked overnight at 4°C in blocking buffer (5 % non-fat dry milk in TBST) before being incubated in primary antibody for 2 h at room temperature (1:1000 in blocking buffer, Hsp72 ADI-SPA-810, Hsp73 ADI-SPA-816, ENZO Life Sciences, Farmingdale, NY, USA). Membranes were then incubated in secondary antibody (1:1000 in TBST, Hsp72 = goat anti-mouse, Hsp73 = goat anti-rabbit, Antibodies Australia, Monash University, VIC, Australia) followed by HRP streptavidin for 30 min (1:1000 in TBST, GTX30949 Genetex,
Inc., Irvine, CA, USA) and then chromagen 3,3’-diaminobenzidine (0.05% DAB, 0.015% H₂O₂, 0.01M PBS, pH 7.2). Membranes were air dried and digitised. Images were used for in-gel band density determination (ImageJ software, National Institutes of Health, Bethesda, USA). Protein abundance was determined by comparing the density of bands from tadpoles in the 28UV- treatment against other treatments.

**Catalase activity**

Catalase is an enzymatic antioxidant which converts H₂O₂ to H₂O + O₂. Catalase activity was determined at three acute test temperatures (18, 23 and 28°C) in a UV/Vis spectrophotometer (Ultrospec 2100 Pro, GE Healthcare, Sydney, Australia). Whole tadpoles (0.018 – 0.164 g) were homogenised in 9 volumes of extraction buffer containing 100mM potassium phosphate buffer (pH 7.4; KH₂PO₄/K₂PO₄), 100 mM KCL and 1 mM ethylene diamine tetra-acetic acid (EDTA). Catalase activity was determined as the decrease in H₂O₂ measured at 240 nm. The assay was conducted in 50mM potassium phosphate buffer (pH 7.8) containing 50 mM of H₂O₂. The extinction coefficient of H₂O₂ is 40 M⁻¹cm⁻¹.

**Oxidative damage**

**Lipid peroxidation**

Lipid peroxidation was determined using a commercially available assay kit which involves the detection of malondialdehyde (MDA), an end product of lipid peroxidation (MAK085, Sigma-Aldrich, Castle Hill, Australia). Lipid peroxidation is determined by the reaction of MDA with thiobarbituric acid (TBA), of which the amount of product (proportional to MDA present) is detected by a colorimetric assay. Briefly, whole tadpoles were homogenised on ice. A 10 mg sample was then homogenised in 300 µl MDA lysis buffer. Supernatant was removed and combined with supplied TBA solution to form the MDA-TBA adduct. This was incubated at 95°C for 60 min before the colorimetric assay was performed in a plate reader (CLARIOstar, BMG Labtech GMBH, Ortenburg, Germany). A separate standard curve was prepared from supplied MDA standard (range 0-20 nmole) and run with each assay.
Protein carbonyl content

Protein oxidation results in the production of carbonyl groups and protein damage was assessed by determining the protein carbonyl content of tissue using a commercially available kit (MAK094; Sigma-Aldrich, Castle Hill, Australia). Briefly, whole tadpole were homogenised in lysis buffer (1:10 w/v; 50 mM imidazole, 2 mM MgCl₂, 5 mM EDTA and 0.1% Triton X-100 pH = 7.5). Following centrifugation 100 μl of supernatant was added to 10μl of 10% streptozocin solution, incubated at room temperature for 10 min and re-centrifuged to remove interfering nucleic acids. Carbonyl content of supernatant was determined by adding 2,4-dinatrophenylhydrazine which leads to the formation of a stable dinitrophenyl hydrazine adduct which was detected spectrophotometrically at 375 nm in a plate reader (CLARIOstar, BMG Labtech GMBH, Ortenburg, Germany). The protein content of each sample was determined using a bicinchoninic acid (BCA) assay (Sigma, Castle Hill, Australia).

Statistics

Catalase activity was analysed using a linear mixed-effects model with temperature treatment, UVR treatment and test temperature included as fixed effects, and tadpole ID nested in tank ID included as a random factor. CTₘₐₓ, lipid peroxidase, protein carbonyl and Hsp72 (heat shocks 31, 34 and 36°C) data were analysed using linear mixed-effects models with temperature and UVR treatment as fixed effects, and tank ID as a random factor. Body mass was included as a fixed effect in the analysis of CTₘₐₓ. Hsp73, Hsp72 (heat shock 28°C) and survival data were analysed using 2-factorial ANOVA to investigate an interactive effect between temperature and UVR as tank was not replicated in these measures. Data were log- transformed where required to meet assumptions of normality. Where indicated, Tukey’s post hoc pairwise analysis was used. Alpha was set as P = 0.05. All analyses were done using the R statistical software package (R Development Team, 2014). Data are presented as mean ± s.e.m.
**Results**

**Survival**

Overall survival was significantly affected by treatment temperature ($F_{2, 32} = 5.11, P = 0.012$), and survival of tadpoles was significantly lower in the 28°C treatment compared to the 18°C treatment (Tukey HSD $P = 0.011$; Figure 4.1). There was no difference in survival of tadpoles between the fluctuating temperature treatment and tadpoles in either cold or warm temperature treatments (Tukey HSD $P = 0.265$ and $P = 0.304$, respectively). There was no interactive effect between temperature and UVR treatment ($F_{2, 30} = 0.73, P = 0.491$), and UVR exposure also did not significantly affect survival ($F_{1, 32} = 2.20, P = 0.148$).

![Figure 4.1](image)

**Figure 4.1.** Survival of tadpoles in temperature and UVR treatments. Tadpoles were acclimated to either 18°C, 28°C or daily fluctuating temperatures (18-28°C) in the presence (grey bars) or absence (white bars) of UVR. Survival was significantly affected by treatment temperature ($F_{2, 32} = 5.11, P = 0.012$), with survival in the 28°C treatment significantly lower than the 18°C treatment (Tukey HSD $P = 0.009$). There was no difference in the survival of tadpoles between the fluctuating temperature (18-28°C) treatment and either 18°C or 28°C temperature treatments. UVR exposure did not significantly affect survival ($F_{1, 32} = 2.20, P = 0.148$). Letters denote significant differences.
There was a significant interaction between temperature and UVR treatment in their effect on CT\textsubscript{max} ($F_{2, 65} = 3.83$, $P = 0.027$). In the high temperature treatment, UVR exposure decreased CT\textsubscript{max}, whereas in the cold temperature treatment, UVR exposure increased CT\textsubscript{max} (Figure 4.2).

**Figure 4.2.** Critical thermal maxima of tadpoles in temperature and UVR treatments. Critical thermal maximum (CT\textsubscript{max}) of tadpoles was affected by a significant interaction between temperature and UVR treatment ($F_{2, 65} = 3.83$, $P = 0.027$). Tadpoles acclimated to 18°C had a higher CT\textsubscript{max} when exposed to UVR (grey bars) compared to tadpoles not exposed to UVR (white bars). Tadpoles acclimated to 28°C had a lower CT\textsubscript{max} when exposed to UVR. UVR did not affect CT\textsubscript{max} for tadpoles in the fluctuating temperature treatment (18 - 28°C).
Heat shock protein abundance

Hsp73

There was no significant effect of treatment on the relative abundance of Hsp73 (Table 4.1, Figure 4.3A).

Hsp72

When exposed to a 28°C heat shock there was no significant effect of treatment on relative abundance of Hsp72 (Table 4.1). Temperature treatment had a significant effect on Hsp72 abundance after exposure to a heat shock of 31°C and 36°C (Table 4.1). Hsp72 abundance was greater in the fluctuating treatment compared to the cold and hot treatment after a heat shock of 31°C. Hsp72 abundance was also significantly greater in the fluctuating treatment compared to the cold treatment after a heat shock of 36°C (Tukey HSD $P < 0.02$ for all comparisons). When exposed to a heat shock of 34°C, exposure to UVR significantly increased abundance of Hsp72 (Table 4.1). Tadpoles from the 18°C/UV+ treatment did not survive the heat shock at 36°C (Figure 4.3B).
Table 4.1. Relative density of Hsp73 and Hsp72 in tadpoles exposed to temperature and UVR treatments. Results from ANOVA (AOV) and linear mixed effects models (LME) for relative density of Hsp73 and Hsp72. Numerator degrees of freedom are shown as subscripts with fixed-effects (Temp, temperature treatment; UVR, UVR treatment) in the source column, while denominator degrees of freedom are shown as subscripts of F-values. Significant effects are shown in bold.

<table>
<thead>
<tr>
<th>Source</th>
<th>Hsp73&lt;sup&gt;AOV&lt;/sup&gt; &lt;br&gt;28°C&lt;sup&gt;AOV&lt;/sup&gt;</th>
<th>Hsp72 &lt;br&gt;31°C&lt;sup&gt;LME&lt;/sup&gt;</th>
<th>34°C&lt;sup&gt;LME&lt;/sup&gt;</th>
<th>36°C&lt;sup&gt;LME&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp&lt;sub&gt;2&lt;/sub&gt;</td>
<td>F&lt;sub&gt;30&lt;/sub&gt; = 1.1153, P = 0.341</td>
<td>F&lt;sub&gt;29&lt;/sub&gt; = 0.4792, P = 0.6241</td>
<td><strong>F&lt;sub&gt;34.99&lt;/sub&gt; = 7.2739, P = 0.002282</strong></td>
<td>F&lt;sub&gt;32.62&lt;/sub&gt; = 3.0517, P = 0.06097</td>
</tr>
<tr>
<td>UVR&lt;sub&gt;1&lt;/sub&gt;</td>
<td>F&lt;sub&gt;1&lt;/sub&gt; = 1.0289, P = 0.3185</td>
<td>F&lt;sub&gt;1&lt;/sub&gt; = 2.4431, P = 0.1289</td>
<td>F&lt;sub&gt;1&lt;/sub&gt; = 0.3886, P = 0.537088</td>
<td><strong>F&lt;sub&gt;1&lt;/sub&gt; = 6.0495, P = 0.01939</strong></td>
</tr>
<tr>
<td>Temp x UVR&lt;sub&gt;2&lt;/sub&gt;</td>
<td>F&lt;sub&gt;2&lt;/sub&gt; = 0.7937, P = 0.4614</td>
<td>F&lt;sub&gt;2&lt;/sub&gt; = 1.6644, P = 0.2069</td>
<td>F&lt;sub&gt;2&lt;/sub&gt; = 0.5812, P = 0.564511</td>
<td>F&lt;sub&gt;2&lt;/sub&gt; = 1.4604, P = 0.24693</td>
</tr>
</tbody>
</table>

Figure 4.3. Western Blot of Hsp73 and Hsp72 abundance. Representative Western Blot of A constitutively expressed Hsp73 and B stress-induced Hsp72 abundance in whole tadpole tissue from tadpoles exposed to one of three temperature treatments (18, 18-28, 28°C) in the absence/presence of UVR (UV-/UV+).
Figure 4.4. Relative density of protein bands for Hsp73 and Hsp72. A Hsp73 was not significantly different between treatments. B Hsp72 was not different between treatments after a heat shock at 28°C. Tadpoles in the daily fluctuating temperature treatment (18 - 28°C) had higher density of Hsp72 compared to tadpoles from cold (18°C) and warm (28°C) acclimation treatments after a heat shock of 31°C (F2,34.99 = 7.27, P = 0.002), and higher density than tadpoles from cold treatments after a heat shock at 36°C (F2,25.28 = 7.80, p = 0.003). After a heat shock at 34°C, tadpoles exposed to UVR (grey bars) had higher Hsp72 density than tadpoles not exposed to UVR (white bars; F1,32.62 = 6.05, P = 0.019) but this was not affected by temperature treatment (F2,32.62 = 3.05, P = 0.06). Tadpoles from the cold temperature treatment did not survive the heat shock of 36°C. Letters denote significant differences.
Catalase activity

Catalase activity was significantly affected by temperature treatment \((F_{2, 51} = 10.65, P < 0.001)\), and activity was significantly higher in tadpoles from the fluctuating temperature treatment compared to tadpoles from either cold or hot temperature treatments (Tukey HSD \(P < 0.02\)). UVR treatment and test temperature did not affect catalase activity (UVR; \(F_{1, 105} = 1.23, P = 0.27\), Test temperature; \(F_{1, 105} = 0.57, P = 0.45\) Figure 4.5). There was no interaction between temperature and UVR treatment \((F_{2, 54} = 0.04, P = 0.948)\).

![Catalase activity graph](image)

Figure 4.5. Catalase activity in *L. peronii* tadpoles acclimated to temperature and UVR treatments. Catalase activity (test temperatures pooled) was significantly higher in tadpoles acclimated to a daily fluctuating temperature treatment (18 - 28°C) compared to tadpoles from cold (18°C) and warm (28°C) acclimation treatments \((F_{2, 51} = 10.65, P < 0.001)\). Catalase activity was not affected by the presence (grey bars) or absence (white bars) of UVR \((F_{1, 105} = 1.23, P = 0.27)\), or test temperature \((F_{1, 105} = 0.57, P = 0.45)\). Letters denote significant differences.

Oxidative damage

Lipid peroxidation

Lipid damage was significantly higher in tadpoles exposed to UVR radiation \((F_{1, 38.97} = 5.40, P = 0.025)\). The degree of lipid peroxidation was not affected by temperature treatment \((F_{2, 38.36} = 0.80, P = 0.459)\) and there was no interaction between temperature and UVR treatment \((F_{2, 70} = 1.07, P = 0.349\), Figure 4.6A).
Protein oxidation

Protein damage was significantly greater in tadpoles exposed to UVR ($F_{1,23.75} = 4.85, P = 0.04$). Treatment temperature did not affect the amount of protein oxidation in tadpoles ($F_{2, 41.88} = 1.03, P = 0.37$) and there was no interaction between temperature and UVR treatment ($F_{2, 41.36} = 0.03, P = 0.97$, Figure 4.6B).

![Figure 4.6](image.png)

Figure 4.6. Oxidative damage in *L. peronii* tadpoles acclimated to temperature and UVR treatments. A Lipid peroxidation ($F_{1,38.97} = 5.40, P = 0.0254$) and B protein oxidation ($F_{1, 23.75} = 4.85, P = 0.04$) were significantly higher in tadpoles exposed to UVR (UV+: grey bars) compared to those that did not receive UVR (UV-; white bars). Temperature treatment did not affect lipid peroxidation ($F_{2, 38.36} = 0.795, P = 0.459$) or protein oxidation ($F_{2, 41.88} = 1.03, P = 0.37$). Letters denote significant differences.

Discussion

Upper thermal limits of *L. peronii* tadpoles were influenced by an interactive effect between temperature and UVR, consistent with similar findings in a related species, *Platyplectrum ornatum* (Kern et al., 2014). Exposure to UVR at cold temperatures increased upper thermal limits, showing that UVR exposure can result in cross-tolerance to high temperatures in some cases. However, contrary to our predictions, exposure to UVR at high temperatures led to a synergistic interaction between stressors, where upper thermal limits were lower in tadpoles in the presence of UVR. Exposure to lethal levels of UVR can lead to detrimental temperature dependent effects for amphibians (Grant and Licht, 1995;
van Uitregt et al., 2007). Our results show that sub-lethal levels of UVR can also have temperature dependent effects on physiological traits through cross-tolerance to high temperature. These results show that although individual stressors, such as temperature, may be important in shaping thermal tolerance, it is likely the interactive effects of multiple environmental factors that shape physiological limits. The role of interactive effects of multiple stressors in determining upper thermal limits may have implications for how species distributions change in the future, especially for those living close to their thermal limits if exposure to additional stressors can compromise physiological tolerances (Duarte et al., 2012; Sinervo et al., 2010). It is important to understand what and how other factors can influence the plasticity of upper thermal limits in order to develop a mechanistic understanding of how environmental variation influences physiological limits and survival.

Our results show that temperature and UVR interact to influence upper thermal limits, but not through any impact on the most likely mechanistic foundations for this (the abundance of Hsp70, oxidative damage or catalase activity). The interactive effect of temperature and UVR on upper thermal limits was not reflected by changes in the abundance of constitutively expressed or stress-induced Hsp70. Constitutively expressed Hsp73 was not affected by temperature or UVR treatment, while expression of induced Hsp72 was affected by temperature treatment and UVR independently at different heat shock temperatures above 28°C. As Hsp72 abundance was higher in tadpoles from the fluctuating temperature treatment, higher expression of Hsp may prevent damage during temperature stress in variable environments (Tomanek, 2010; Lopez-Martinez and Denlinger, 2008). Alternatively, the heat shock temperatures used may have failed to induce this stress response in tadpoles from the 28°C treatment, which may explain why protein abundance in this group did not reflect their increased CT_max. While Hsp70 expression may in some cases be correlated with upper thermal limits (Fangue et al., 2011; Kalosaka et al., 2009), abundance of this protein does not always predict plasticity of this trait (Healy and Schulte, 2011; Dahlgaard et al., 1998), highlighting the difficulty in understanding the mechanistic basis of complex physiological traits. Furthermore, the independent effects of temperature and UVR on the abundance of Hsp72 illustrate that understanding the effects of single stressors on mechanistic traits may not explain complex pathways at the cellular level which result in interactive effects at the whole-animal level.
Tadpoles exposed to UVR suffered increased oxidative damage of proteins and lipids, but this damage was not influenced by acclimation temperature. This suggests that antioxidant activity was not impeded at cold temperatures, since damage was not increased in tadpoles from the 18°C temperature treatment. Catalase activity was not influenced by acclimation to the cold or hot temperature treatments, but acclimation to a fluctuating temperature regime did increase the activity of catalase. Due to the lack of a mean temperature control for the fluctuating treatment (i.e. constant 23°C) we cannot determine whether the increase in catalase activity was associated with anticipation of high temperatures in variable environments (Tomanek, 2010; Lopez-Martinez and Denlinger, 2008), daily exposure to high environmental temperatures (Lalouette et al., 2011), or in response to the mean temperature signal. The increased activity of catalase in the fluctuating treatment however, was not enough to buffer tadpoles from the additional oxidative damage caused by UVR. Interestingly the activity of catalase was not affected by acute temperature change over the range tested, suggesting a low activation energy of this enzyme in this species (Daniel et al., 2010; Laidler and Peterman, 1979). The temperature independent activity of catalase in this species differs from a number of other species which show acute thermal sensitivity of this enzyme and the capacity to acclimate activity (Gil and Barja de Quiroga, 1988; Kaushik and Kaur, 2003; Morgulis et al., 1926; Selman et al., 2000). Low thermal sensitivity of catalase activity may be beneficial in variable environments if activity can be maintained without the costs associated with acclimation (DeWitt et al., 1998). Defences against oxidative damage are multifaceted, involving many enzymatic and non-enzymatic components (Turrens, 2003). Further investigation of the specific effects of UVR and temperature on cellular mechanisms may reveal more about what makes this species robust to temperature induced oxidative damage.

Upper thermal limits, and stress tolerance in general are complex traits which likely result from the cumulative action and expression of many different Hsps and numerous other mechanisms (Feder and Hofmann, 1999a; Overgaard et al., 2014). Our results highlight the difficulties in predicting the responses of even well understood mechanistic traits (i.e. Hsps) to environmental stress. Although we understand the function of many cellular mechanisms and their role in animal physiology, we are still lacking in our understanding of the mechanistic causes underlying thermal limits (this study, Healy and Schulte, 2012). Continuing to focus on individual mechanistic traits may fail to explain the physiological responses of animals to environmental variation. Even if we were able to establish a
detailed understanding of all of the molecular mechanisms involved in responding to temperature and UVR exposure, the additive effects of all these mechanisms may still not completely explain the plasticity of higher level traits due to the potential of emergent properties in biological systems (Allen and Polimene, 2011; Jablonka and Lamm, 2012). Complex networks and interactions of numerous proteins are likely to be involved in regulating plasticity and trait variation. Determining what drives the interactive effect of temperature and UVR exposure on upper thermal limits may not be fully explained by understanding the response of lower level traits only, and may require a systems based approach to understand how different levels of organisation contribute to whole animal traits (Feder and Walser, 2005; Fly and Hilbish, 2013; Suarez and Moyes, 2012).

These findings highlight not only the importance of multiple stressor studies for our understanding of an organisms’ physiological response to environmental variability, but also the complexity of the mechanistic underpinnings to these responses. The lack of a direct relationship between the effects of temperature and UVR on upper thermal limits and the cellular mechanisms investigated here illustrates that other mechanisms or the cumulative action of multiple stress response mechanisms must be responsible for the effects on upper thermal limits. While it is now increasingly apparent that the outcomes of multiple stressor studies cannot be predicted from the results of single stressor studies, understanding the mechanistic basis for whole-animal level responses to interacting stressors is equally complicated by interactions between and among complex cellular response pathways. Similarly, the underlying basis for whole-animal level responses to multiple stressors may not be predictable from our understanding of the mechanisms behind responses to any single stressor. A greater understanding of the range of responses initiated by exposure to UVR may reveal the mechanistic basis for cross-tolerance to high temperature, and go some way to explaining the interactive effects demonstrated. It is necessary to consider the interactive effects of multiple environmental factors on animal physiology at all levels of biological organisation in order to develop our understanding of how environmental tolerances are shaped.
Species responses to environmental variation are complex as they are determined by a number of factors including the time scale of variation and the interactive effects of multiple stressors. Temperature and UVR are key environmental drivers that increase energetic demands and can induce plasticity in mechanistic traits, determining tolerance to environmental stress. Importantly temperature and UVR have interactive effects on morphological and physiological traits, and survival. The overarching aim of this thesis was to investigate physiological responses to diel thermal fluctuations and exposure to UVR in anuran tadpoles.

Temperature is a particularly important factor influencing the physiology of ectotherms, and the capacity to alter physiological rate processes can buffer energetic demands in variable environments. Ectotherms experiencing DTFs would benefit from reducing the thermal sensitivity of physiological traits in order to reduce the energetic costs associated with time spent at high temperatures, and to maintain performance over the course of the day. My research showed that tadpoles were unable to alter thermal sensitivity of physiological traits (Chapter 2), which led to reduced growth and altered the length of development in the species studied. Daily thermal fluctuations led to longer development times in *Limnodynastes* species and shorter development times in *P. ornatum* reflecting the species specific responses that can be induced by DTFs (Dhillon and Fox, 2007; Les et al., 2009; Folguera et al., 2011). Fluctuating temperatures also increased upper thermal limits, making tadpoles more tolerant to high temperature stress. While increased stress resistance may buffer animals from lethal consequences of temperature extremes, the costs of DTFs on growth and development are likely to negatively impact species fitness (see Significance of Research).

When animals experience temperature variation repeatedly within their lifetime selection should favour the capacity to reversibly modulate physiological processes to match changed conditions (Levins, 1968; Angilletta, 2009). Ectotherms inhabiting highly variable environments are predicted to develop broad thermal performance curves (TPCs) which encompass the range of temperatures experienced (Huey and Hertz, 1984; Ruel and Ayres, 1999). As such, plasticity in response to DTFs would not be advantageous if a
broad and fixed TPC allows individuals to maintain performance across the range of temperatures experienced. Conversely, individuals from less thermally variable environments, with narrower TPCs may be expected to reduce thermal sensitivity of traits in response to DTFs (Měráková and Gvoždík, 2009; Williams et al., 2012). The results from Chapter 2 do not support this hypothesis as the amount of thermal variation in the environment inhabited by related species did not predict the capacity of individuals to flexibly alter physiological or performance traits in response to DTFs. Instead all species investigated lacked the capacity to acclimate thermal sensitivity of traits to DTFs (Chapter 2 and 3). *Limnodynastes* tadpoles appear to have responded to stable cues of the temperature regime and increased the activity of metabolic enzymes in response to the cold overnight temperatures (modal temperature in this case). Investigating the relative importance of different cues by including temperature treatments which varied in their mean, mode and thermal amplitude offers the opportunity to reveal more about the reliability of cues in variable environments.

Variation in TPCs and acclimation capacity is not necessarily shaped by selective pressures caused by environmental variation and may be genetically constrained by the phylogenetic histories of different populations/species (Bonino et al., 2011; Seebacher et al., 2012). Phylogenetic effects may explain why the responses to DTFs reported in Chapter 2 were species specific. In future, investigating the role of environmental variation in shaping acclimation responses should use phylogenetically independent contrasts (Bonino et al., 2011), or conspecifics from different populations to better account for genetic constraints. Populations of one species inhabiting environments which differ in the amount of DTFs may be a better system to investigate the influence of environmental variation on the capacity to acclimate in response to DTFs (Williams et al., 2012). It is important to establish when individuals can reduce the thermal sensitivity of traits in order to understand species responses to DTFs.

The presence of additional stressors can alter phenotypic outcomes of temperature variation. The results from Chapter 3 demonstrate interactive effects of temperature and UVR on tadpole survival which strengthens previous findings of temperature dependent effects of UVR exposure (Grant and Licht, 1995; Hoffman et al., 2003; van Uitregt et al., 2007). Cold temperatures increase the lethal consequences of UVR exposure (Grant and Licht, 1995; Hoffman et al., 2003; van Uitregt et al., 2007). My research demonstrated that DTFs can also increase the lethal consequences of exposure to UVR (Chapter 3). Based
on previous studies, it was predicted that effects of UVR exposure on growth and development would be associated with increased maintenance costs caused by UVR damage repair (Formicki et al., 2003; Alton et al., 2012; Cramp et al., 2014). As there was no effect of UVR exposure on whole animal resting metabolic rate (Chapter 3) increased maintenance costs may not be responsible for the interactive effects observed on tadpole survival. Instead, the interactive effects of temperature and UVR on survival may be caused by direct and indirect damage resulting from exposure to UVR, and the thermodynamic effects on repair mechanisms.

In this thesis I demonstrated for the first time that temperature and UVR interact to determine upper thermal limits. The scale of temperature variation influenced outcomes of plasticity on upper thermal limits with both mean temperature (Chapter 4) and DTFs (Chapter 3) affecting consequences of UVR exposure. Both short-term temperature fluctuations and changes in UVR levels are therefore important in determining resistance to increasing temperatures. The interactive effects of temperature and UVR exposure on physiological tolerance were predicted to reflect changes to lower level mechanistic traits. The results in Chapter 4 showed that the interactive effects of temperature and UVR on upper thermal limits were not explained by the induction of Hsp70 and other mechanistic traits. The indirect consequences of UVR exposure increased oxidative damage through the production of ROS, although again this, nor the activity of an antioxidant enzyme catalase, were temperature dependent (Chapter 4). The causes of interactive effects of multiple stressors are complex, and may not be the direct results of changes in a number of mechanistic processes.

Temperature and UVR are pervasive environmental drivers that modify growth, development and physiological stress tolerance. Investigating physiological responses to different scales of temperature variation reveal more about how animals function in variable environments. Furthermore, the consequences of temperature variability can be altered by the presence of additional stressors such as UVR, and these interactive effects are important in developing our understanding of how animals function in complex environments. Integrative approaches assessing mechanistic and whole animal traits develop our understanding of what drives responses to variable environmental conditions.
Significance of Research

Understanding how individual animals respond to environmental variation is crucial in order to predict population persistence in the face of environmental change. The findings of this research have significant implications for our understanding of how animals respond to environmental variation;

1. Species specific responses dictate the consequences of daily thermal variability
2. Responses to thermal variability at the individual level (survival, rate of development, body size) determine biotic interactions and population level responses
3. Diurnal thermal variation can reveal energetic consequences of additional environmental stressors
4. Interactive effects of multiple environmental variables determine physiological tolerance to environmental stress
5. Complex responses to environmental variation may not be explained through effects on mechanistic traits

The significance of these findings is discussed below.

Daily thermal fluctuations influence metabolic demands altering growth rates, development and survival. The results presented in Chapter 2 support previous findings that the energetic consequences of DTFs and effects on development are highly variable between species (Dong et al., 2006; Dhillon and Fox, 2007; Du and Shine, 2010; Folguera et al., 2011). The species investigated had little capacity to reduce energetic consequences of DTF, leading to reductions in growth (Chapter 2 and 3). Reduced body size as a consequence of increased thermal variability negatively affects anuran fitness by increasing predation risk during and after metamorphosis as well as reducing fecundity at maturity (Sweeney and Schnack, 1977; Wilson et al., 2000b; Kingsolver and Huey, 2008). Furthermore, different developmental responses to DTFs between species can alter survival. In semi-permanent aquatic environments increased thermal variability is associated with deteriorating conditions. Tadpoles which are unable to increase development (Chapter 2, *Limnodynastes sp.*) will suffer high mortality if they are unable to metamorphose before habitats dry. *P. ornatum* tadpoles increased development in response to DTFs (Chapter 2), which may reflect an adaptation to thermally variable
conditions due to their ecological niche. Increased variability associated with climate change (IPCC, 2013) may therefore favour species that are able to increase development, or already have rapid development. Species from less thermally variable habitats that cannot compensate for increased variability will be most affected. If adaptation to certain conditions infers survival, responses to climate change may be determined by the niche occupied rather than the capacity for plasticity.

Species specific responses to temperature variation are important in determining community interactions. The capacity to mitigate fitness consequences of thermal variation are species specific (Chapter 2; Dong et al., 2006; Niehaus et al., 2006; Měráková and Gvoždík, 2009; Folguera et al., 2011), and will therefore influence biotic interactions which result from the combination of individual performance. In Chapter 2 tadpoles were unable to reduce the thermal sensitivity of performance, and as a consequence predator avoidance behaviour was compromised at cold temperatures. This will influence predator-prey interactions. As predator activity is also temperature dependent biotic interactions will be determined by both species responses to temperature variability (Grigaltchik et al., 2012). As tadpole performance was reduced at cold temperatures, predation pressure may increase when water temperatures are low, especially if predators have a greater capacity to flexibly alter physiology and maintain performance across diurnal temperature fluctuations. Different capacities of predators and prey to alter upper thermal limits will also alter predation pressure at high temperatures. Predator-prey interactions will vary with environmental variation as species specific acclimation capacity, and innate thermal sensitivity dictate these relationships. Complex relations may develop as predator presence itself influences morphology and physiology of prey (Relyea, 2001; Alton et al., 2010). Furthermore, phenological changes resulting from temperature effects on development may create a mismatch in biotic interactions (Durant et al., 2007; Traill et al., 2010). For example, delayed emergence of metamorphs from developmental pools may coincide with reduced food availability if their invertebrate prey have already reproduced or migrated. Understanding individual responses to environmental variation is critical in understanding changes to biotic interactions.

Most environments experience some degree of DTF (Paaijmans et al., 2013) and this scale of temperature variability will have effects at the individual and population level (Estay et al., 2014; Vasseur et al., 2014; Lawson et al., 2015). Models suggest that fluctuating conditions will influence population growth rate depending on the shape of the
growth curve (Roland and Matter, 2013; Estay et al., 2014; Lawson et al., 2015). However, population level change may be determined by the impacts of temperature fluctuations on individuals. For example, slower development (Chapter 2 and 3, Niehaus et al., 2012; Arrighi et al., 2013) results in longer generation time, while smaller body size at maturity may reduce population recruitment due to increased susceptibility to predation (Wilson and Franklin, 2000; Kingsolver and Huey, 2008), and fewer offspring resulting from reduced fecundity (Sweeney and Schnack, 1977; Carroll and Quiring, 1993). Furthermore DTFs alter immune function and disease dynamics (Paaijmans et al., 2010; Lambrechts et al., 2011; Raffel et al., 2013; Terrell et al., 2013), and shape physiological tolerances and survival when exposed to environmental stress (Chapter 2 and 3; Feldmeth et al., 1974; Ravaux et al., 2012). The cumulative impacts of DTF at the level of the individual are important in determining population responses to increased thermal variability.

Daily temperature fluctuations can determine the effects of other environmental variables. In Chapter 3, UVR exposure reduced the survival of tadpoles experiencing DTFs, but not those at constant temperature although treatments means were equivalent. The species investigated, *P. ornatum* inhabits highly thermally variable environments, and as such conclusions drawn about the impacts of UVR at stable temperatures will not reflect ecological consequences of UVR exposure. Fluctuating thermal environments impose different energetic challenges than equivalent mean temperature conditions (Chapter 2; Niehaus et al., 2012; Arrighi et al., 2013) and produce thermodynamic effects on physiological traits. As such, DTFs plays an important role in shaping physiological responses. This is illustrated by the consequences of parasitoids on their insect hosts being modulated by DTFs (Kitano, 2002; Cramp et al., 2014), and amphibian immune defenses being more robust in DTFs compared with mean temperature conditions (Terrell et al., 2013). Our understanding of what shapes physiological responses to environmental variation will benefit from incorporating different scales of temperature fluctuations.

Interactive effects of environmental stressors shape physiological tolerances. In Chapter 4, the interactive effect of high temperatures and UVR reduced upper thermal limits. If thermal tolerance is reduced by UVR exposure, changing levels of UVR in concert with increased temperature will determine species outcomes in response to climate change. Many biotic and abiotic stressors lead to plasticity in thermal tolerance, including diet (Jirsa et al., 2013), presence of predators (Sørensen et al., 2011), environmental contamination (Pandolfo et al., 2010), UVR (Chapter 3 and 4) and precipitation (Clusella-Trullas et al.,
If these stressors also interact with temperature to affect thermal tolerances, natural and anthropogenic environmental variation may alter survival. For example, environmental disturbances can increase species exposure to predations and pollutants as well as altering the thermal environment. If temperature modulates the effects of predator pressure or pollutants on upper thermal limits, individuals’ tolerance to high temperatures may be reduced. As upper thermal limits are used to predict the persistence of species under climate change (Sinervo et al., 2010; Tomanek, 2010; Duarte et al., 2012) it is crucial to understand how environmental stressors interact to influence plasticity of this trait.

Thermal tolerance is determined by the interaction of underlying mechanistic traits (Feder and Hofmann, 1999; Kitano, 2002). The interactive effects of temperature and UVR on upper thermal limits were not explained by the mechanistic traits investigated in Chapter 4. Predicting changes in whole animal traits from mechanistic traits is difficult due to emergent properties of complex biological systems (Kitano, 2002; Feder and Walser, 2005). It is increasingly appreciated that whole animal traits may not simply be the sum of mechanistic traits, but that interactive effects of multiple cellular processes produce unexpected results at higher levels of organisation (Feder and Hofmann, 1999). As the effects of multiple stressors may not be explained by the additive consequences of individual stressors, the same may be true for complex biological systems where the phenotype is not predicted by the sum of all of its components (Poisot et al., 2011). For example, networks between Hsps produce emergent properties which influence immune function (Nardai et al., 2006). Similarly, complex associations between cellular protective mechanisms (i.e. oxidative damage to Hsps during UVR exposure [Sóti and Cseméry, 2002]) may lead to emergent properties that contribute to the interactive effect of temperature and UVR on upper thermal limits demonstrated in this thesis (Chapter 3 and 4). If the interactive effects of multiple stressors on whole animal traits cannot be predicted by lower level traits, systems based approaches need to be employed.
Future Directions

This thesis has extended our understanding of developmental consequences of temperature variability and exposure to UVR. Species responses to temperature variability will likely depend on individual capacities to overcome energetic challenges, and physiological tolerance to temperature may be determined by the interactive effects of multiple stressors. The key conclusions from this thesis highlight the importance of understanding what drives physiological responses to environmental variation, and raises a number of questions that could direct future research.

How do animals respond to unpredictable temperature variation?

This thesis examined physiological responses to predictable levels of DTFs. Forecasts predict an increase in frequency of extreme weather events and unpredictability of environmental conditions (Easterling et al., 2000; IPCC, 2013). Understanding how animals are affected by unpredictable thermal variation is important for determining the energetic and physiological consequences of variable environments and predicting species responses to climate change. Unpredictably variable conditions produce different outcomes from predictably variable conditions through changes in energetic consequences and effects on physiological traits (Thorp and Wineriter, 1981; Munn et al., 2010). For example, *Drosophila simulans* exposed to predictable and unpredictable fluctuating temperatures (equal in mean temperature and thermal amplitude) resulted in flies exposed to unpredictable temperature fluctuations having significantly reduced stress resistance (Dahlhoff, 2004). Similarly, unpredictably variable food availability led to greater use of an energy saving mechanism (torpor) than a diet of equal energy content delivered at a constant rate (Munn et al., 2010). More frequent high temperature extremes may increase the rate at which dormant animals consume energy reserves (Young et al., 2011) reducing survival in inclement years. Investigating the consequences of unpredictable temperature variation, will reveal more about how animals respond to another scale of environmental variation.

Phenotypic plasticity may not develop in response to unpredictable temperature fluctuations if individuals are not able to perceive cues about future environmental conditions. Current experimental cues which induce plasticity (i.e. mean, amplitude of variation, maximum) are insufficient for any meaningful insight into the effects of increased
unpredictability on the physiology of ectotherms. Our understanding of how unpredictably variable conditions will alter TPCs is limited (Canale and Henry, 2010), although tools to predict the consequences of these conditions are being developed (Botero et al., 2015). Using field collected temperature data may be one way to incorporate ecologically relevant variation into investigations of plasticity (Williams et al., 2012). Unpredictability of environmental conditions needs to be investigated in order to fully understand physiological responses to current and future environmental variation.

**How do communities respond to complex ecological scale variation?**

The results of this thesis demonstrated species specific responses to DTFs which were altered by the presence of other environmental stressors. Species specific responses to environmental variation will be instrumental in determining biotic interactions. Investigating how individual capacities to thermally acclimate influence competitor and predator-prey interactions will be important in determining population responses to environmental variation. For example, determining the thermal sensitivity of tadpoles and their predators (i.e. dragonfly larvae) after acclimation to DTFs, and observing their interactions during diurnal temperature change would reveal how fitness consequences for both are moderated by having or lacking the capacity to flexibly alter performance traits in these conditions. The next step would then be to determine how the interactive effects of DTFs and other stressors influence these interactions.

In ephemeral aquatic environments, many variables influence growth, development, performance, and the plasticity of these traits. Conspecific density, water volume and predator presence are a few biotic and abiotic variables that can induce plasticity in tadpole growth and performance (Alton et al., 2010; Szekely et al., 2010; Mitchell et al., 2012). The interactive effects of all these variables are likely to be complex, and phenotypic effects demonstrated in controlled experiments manipulating one or two of these may misrepresent ecological responses to environmental variation. Predicting how multiple variables will change as a consequence of future environmental variation is difficult (Canale and Henry, 2010) and complicates how future environments should be dealt with in experimental design. Complex multifactorial studies may be best suited to semi-natural experimental enclosures through the use of mesocosms. These systems better replicate environmental variation (i.e. diurnal fluctuation, predator presence, conspecific density and competition) while enabling manipulation of variables of interest, or
examination of particular species interaction (Losey and Denno, 1998; Keller and Klein-MacPhee, 2000; Boone and James, 2003). For example, temperature could be manipulated with heaters, and UVR levels determined by the use of UVR retardant film. Furthermore, species interactions could be recorded in situ. Mesocosms allow investigation of specific variables, while accounting for the phenotypic effects of additional stressors, and stressor interactions. This type of approach would further our understanding of how interactive effects influence the plasticity of traits and what shapes physiological tolerances of organisms (Heckmann et al., 2008).

What is the mechanistic basis for the interactive effects of temperature and UVR?

To further examine mechanistic causes of the interactive effects of temperature and UVR exposure on survival it is important to investigate how temperature influences the efficiency of mechanisms which repair UVR damage. UVR directly damages DNA by forming lesions which distort the DNA helix, resulting in inefficiencies in transcription and replication that can result in cell death (MacFadyn et al., 2004). The repair of UVR induced DNA damage involves multi-step enzymatic repair mechanisms such as nuclear excision repair (NER), and a light dependent (UV-A or visible wavelengths 320 – 700nm) enzyme mediated photo-enzymatic repair (PER, Sinha and Hader, 2002; Friedburg et al., 2006). The capacity to repair UVR damage underlies species susceptibility to UVR exposure (Blaustein et al., 1994). These major UVR damage repair mechanisms have been shown to be temperature sensitive (Langenbacher et al., 1997; Pakker et al., 2000; MacFadyn et al., 2004; Sanders et al., 2005), although no investigations have linked these mechanisms to higher level interactive effects of temperature and UVR. The interactive effects of temperature and UVR on survival demonstrated in Chapter 3 and other studies (Grant and Licht, 1995; van Uitregt et al., 2007) may be explained by the thermal sensitivity of these major repair mechanisms, and the inability to repair damage at cold temperatures. Examining how temperature affects the efficiency of NER and PER to repair DNA damage would provide further insights into the mechanistic cause of temperature and UVR interactions.
Will a biological systems approach better enable us to determine what drives physiological tolerances to environmental stress?

It is important to understand how interactions of environmental variables shape plasticity of physiological tolerances in order to understand how animals respond to changes in their environment. Investigating the components involved in initiating stress responses, reveal a large number of genes and proteins which are upregulated in response to temperature stress (Heikkila, 1993; Podrabsky and Somero, 2004; Gracey et al., 2008; Tomanek and Zuzow, 2010). These mechanistic traits can be used to investigate changes in physiological tolerance, but as we demonstrated in Chapter 4 changes in lower level traits may not predict responses at the whole animal level. These approaches may fail to generate a comprehensive understanding of how animals respond to environmental variation by not investigating the properties of the complex networks between the components that determine whole animal responses (Kitano, 2002; Heckmann et al., 2008). In order to understand plasticity in physiological tolerances we need to understand the role of each component involved in responding to stressors through their complex associations with other parts of the stress response system (Cramer et al., 2011). Systems approaches can integrate transcriptomics, metabolomics and proteomics to understand how environmental stressors interact to induce plasticity in physiological tolerances, and can determine the cause of stressor interactions on these traits (Cramer et al., 2011; Suarez and Moyes, 2012). As temperature has numerous and complex actions on different levels of organisation (Hochachka and Somero, 2002), a systems approach may identify where temperature is affecting mechanistic traits, and how the interactions between these traits result in whole organism responses (Cramer et al., 2011). Furthermore, these approaches allow an understanding of how these responses proceed over time, rather than the snap-shot that is achieved with traditional molecular techniques. Systems approaches would greatly improve our understanding of how animals respond to environmental variation and enable better predictions of individual and population level responses.


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