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Title: High temperature, oxygen, and performance: Insights from reptiles and amphibians

Running Title: Hierarchical Mechanisms of Thermal Limitation

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Synopsis Much recent theoretical and empirical work has sought to describe the physiological mechanisms underlying thermal tolerance in animals. Leading hypotheses can be broadly divided into two categories that primarily differ in organizational scale: 1) high temperature directly reduces the function of subcellular machinery, such as enzymes and cell membranes, or 2) high temperature disrupts system-level interactions, such as mismatches in the supply and demand of oxygen, prior to having any direct negative effect on the subcellular machinery. Nonetheless, a general framework describing the contexts under which either subcellular component or organ system failure limits organisms at high temperatures remains elusive. With this commentary, we leverage decades of research on the physiology of ectothermic tetrapods (amphibians and non-avian reptiles) to address these hypotheses. Available data suggest both mechanisms are important. Thus, we expand previous work and propose the Hierarchical Mechanisms of Thermal Limitation (HMTL) hypothesis, which explains how subcellular and organ system failures interact to limit performance and set tolerance limits at high temperatures. We further integrate this framework with the thermal performance curve paradigm commonly used to predict the effects of thermal environments on performance and fitness. The HMTL framework appears to successfully explain diverse observations in reptiles and amphibians and makes numerous predictions that remain untested. We hope that this framework spurs further research in diverse taxa and facilitates mechanistic forecasts of biological responses to climate change.

Introduction

For most animals, the proximate mechanisms that underlie reduced performance and eventual death at high temperatures, and how such mechanisms might change with ontogeny and context, are uncertain (reviewed in Angilletta 2009; Clark et al. 2013; Pörtner et al. 2017). Such a mechanistic understanding would greatly enhance our ability to predict the effects of realistic high-temperature exposures on individuals or populations, which are difficult to forecast with traditional methods because responses can be highly variable (e.g. Gunderson and Stillman 2015; Seebacher et al. 2015; Kingsolver and Woods 2016; Sheldon and Dillon 2016; Williams et al. 2016). Even so, much progress has been made toward developing mechanistic models that use knowledge of individual physiology to predict the effects of thermal environments on populations, largely inspired by growing concern over the impacts of global climate change (Buckley 2008; Kearney and Porter 2009; Huey et al. 2012; Kearney 2012; Levy et al. 2015; Malishev et al. 2017). Commonly, such models employ empirically-derived thermal performance curves (TPC, Huey and Stevenson 1979) to predict the effects of thermal environments on organisms and populations (e.g. Colwell et al. 2008; Deutsch et al. 2008; Vasseur et al. 2014; Buckley and Huey 2016; Dillon et al. 2016). TPCs describe how performance varies with temperature— typically performance increases with temperature above a critical minimum (CT_{MIN}) until an optimum is reached (T_{OPT}), then rapidly drops to zero at the critical maximum (CT_{MAX} , see Fig. 1 for examples). Pejus (getting worse) temperatures above and below T_{OPT} are described by breakpoints in physiological function (e.g. ventilation rate, heart rate, P_{O_2}) indicative of rapid declines in whole-organism performance (T_{PEJUS} , Frederich and Pörtner 2000; Pörtner 2002; Pörtner et al. 2017). Typically TPCs are estimated under controlled laboratory conditions for a single trait and time, but TPC shape can vary with season, ontogeny, trait, and

prior experience in ways that are difficult to predict (Rezende et al. 2014; Telemeco 2014; Kingsolver and Woods 2016; Williams et al. 2016). Thus, using empirically-derived TPCs to predict the effects of natural environments on performance is problematic because it requires extrapolating from the traits or environments originally used to estimate TPCs, thereby ignoring probable context-dependency. A mechanistic understanding of the processes that underlie thermal performance is needed to predict the effects of variable or novel environments on TPC shape, which will greatly improve models relying on TPCs to predict population responses. In particular, knowledge of the mechanisms that result in loss of function at high temperatures is needed to predict the rate at which performance will drop in response to thermal challenge, the capacity for animals to recover from sub-lethal thermal exposure, and the capacity for thermal tolerance to change via plasticity or evolution (Helmuth et al. 2005; Buckley and Huey 2016; Williams et al. 2016).

Potential mechanisms explaining why animals lose function at high temperatures can be divided into two major categories differing in the level of organization first affected. At lower levels of organization, subcellular components could be critically impaired when animals are exposed to temperatures above the optimum. Subcellular impairment results from either denaturation of key molecules, such as proteins and cell membranes, or reduced efficiency of these molecules to perform their biological functions (reviewed in Hochachka and Somero 2002; Angilletta 2009; Schulte 2015). Impairment of subcellular components would result in the breakdown of higher levels of organization and lead to rapid performance loss. Alternatively, higher-levels of organization, such as organ systems, could be impaired at temperatures below those that directly affect the performance of their subcellular components if high temperatures disrupt subcellular interactions or pathways necessary for organ system function. A recent

mechanistic model explaining organ-system impairment at high temperatures is the oxygen and capacity limited thermal tolerance (OCLTT) hypothesis, which proposes that oxygen demand for aerobic metabolism at high temperatures outpaces the ability of the cardiovascular and respiratory systems to provide sufficient amounts of oxygen to tissues (Pörtner 2002; Pörtner and Knust 2007; Verberk et al. 2016; Pörtner et al. 2017). Under this hypothesis, oxygen diffusion and transport capacity limit metabolic rates such that resting rates converge with potential maximum rates at high temperatures thereby reducing aerobic scope to zero (i.e. zero aerobic power budget sensu Pörtner et al. 2017). This mismatch in supply and demand will first reduce aerobic performance at T_{PEJUS} but eventually result in basal oxygen demands not being met and thus collapse of organismal systems at CT_{MAX} . Proponents of OCLTT argue that this mechanism integrates across levels of organization from systemic to molecular mechanisms and can explain diverse evolutionary and ecological phenomena (Pörtner 2002, Pörtner et al 2017). However, the temporal window, activity range, magnitude, and biological relevancy of reduced aerobic scope at high temperature are debated (Clark et al. 2013; Gräns et al. 2014; Jutfelt et al. 2014; Pörtner 2014; Verberk et al. 2016; Jutfelt et al. 2018). Identifying the level of organization first impaired by high temperature is necessary to understand how temperature affects whole-organism function.

Recent work exploring the mechanisms underlying thermal tolerance in ectotherms focuses on aquatic taxa (e.g. Frederich and Pörtner 2000; Lucassen et al. 2006; Verberk et al. 2013; Gräns et al. 2014; Ern et al. 2016; Pörtner and Gutt 2016; Verberk et al. 2018) and terrestrial arthropods, particularly insects (reviewed in McCue and De Los Santos 2013; Verberk et al. 2016). These observations suggest that subcellular-level mechanisms are more important determinants of thermal tolerance than oxygen capacity limitations in terrestrial taxa, perhaps

because oxygen is more abundant in air than water. However, recent work includes few studies of terrestrial vertebrate ectotherms: non-avian reptiles (hereafter, reptiles) and amphibians. Reptiles and amphibians display varied ecology, life-history, and thermal tolerance (Vitt and Caldwell 2009; Sunday et al. 2011) and are important to a broad understanding of thermal tolerance. Additionally, these animals frequently contend with challenging temperatures, with some species inhabiting the hottest terrestrial environments (Cowles and Bogert 1944; Vitt and Caldwell 2009; Sunday et al. 2014). Moreover, both the thermal and oxygen environment can vary throughout ontogeny (e.g. aquatic to terrestrial transition in amphibians), and modes of respiration can vary (e.g. cutaneous respiration in many amphibians and some reptiles, such as turtles; Hutchison et al. 1968; Glass et al. 1981; Wang 2011). Because of these traits, reptiles and amphibians have been important models for thermal physiology and ecology for the last century (e.g. Cowles and Bogert 1944; Snyder and Weathers 1975; Huey and Stevenson 1979; Huey 1982; Huey and Berrigan 2001; Vitt and Caldwell 2009; Kearney 2012). This historic work can be leveraged to address modern ideas, such as the OCLTT hypothesis. Furthermore, these taxa are at risk of high-temperature induced extinction resulting from global climate change (Thomas et al. 2004; Huey et al. 2010; Rohr and Raffel 2010; Sinervo et al. 2010; Kearney 2013). Here, we apply the rich history of physiological studies in reptiles and amphibians to modern hypotheses for mechanisms underlying thermal performance and tolerance. This will complete taxonomic coverage of recent reviews, and, more importantly, further understanding of how organismal performance and survival are restricted by high temperatures.

In this commentary, we begin by synthesizing evidence for subcellular- and organ-system-level failures underlying high temperature tolerance and performance in reptiles and amphibians. Because neither framework satisfactorily explains observed patterns individually,

we next build upon work in other taxa to present an integrative framework combining both subcellular and organ-system sensitivity to high temperatures and the thermal performance curve paradigm. Using data for reptiles and amphibians, we illustrate how this combined framework appears to predict a broad array of physiological and behavioral observations. We conclude by identifying future research directions to test this framework and discuss how it might be applied to other systems to predict consequences of future high-temperature exposure for organisms and populations.

Evidence for mechanisms underlying thermal tolerance and performance in reptiles and amphibians

Subcellular mechanisms

High temperatures compromise the structure and function of subcellular components, such as enzymes and cell membranes (Fields 2001; Hochachka and Somero 2002) but such damage will only underlie organismal thermal tolerance if it occurs at temperatures below those affecting function at higher levels of organization, such as organ systems (Pörtner 2002). The complexities of enzymatic interactions make extending observations for individual reactions to the whole cell or organism potentially problematic (Schulte 2015). Nonetheless, the observation that subcellular components are frequently stable to temperatures above the critical and lethal limits of animals (CT_{MAX} and T_{LETHAL} hereafter), commonly maintaining function to temperatures $> 50^{\circ}C$ and capable of evolving stability to $120^{\circ}C$ (Fields 2001), was an important motivation for the development of hypotheses such as OCLTT (Pörtner 2001; 2002; Pörtner et al. 2017). For example, CT_{MAX} in reptiles and amphibians generally ranges from the mid 30s to low 40s $^{\circ}C$

(Brattstrom 1965; Sunday et al. 2011; Sunday et al. 2014), although a few warm-adapted reptiles such as the desert iguana (*Dipsosaurus dorsalis*) tolerate acute exposure to 47.5 °C (Cowles and Bogert 1944; Brattstrom 1965). Even so, most reptile and amphibian proteins do not lose function until they experience temperatures well above CT_{MAX} . For example, ribonucleases from three frog species were stable to 85°C and maintained high activity at temperatures > 50°C (Irie et al. 1998), lactate dehydrogenase in *Agama stellio* lizards maintained both stability and function to 70°C (Al-Jassabi 2002), and alkaline phosphatase maintained high function up to 50°C in four lizard species (Licht 1964). Similarly, acute exposure to CT_{MAX} did not cause tissue damage or reduce function of serum glutamic-oxaloacetic or glutamic-pyruvic transaminases in great plains toads (*Anaxyrus cognatus*, Paulson and Hutchinson 1987) and 2.5-h exposure to temperatures just below CT_{MAX} had no effect on mitochondrial respiration or free-radical production in alligator lizards (*Elgaria coerulea* and *E. multicaerinata*; Telemeco et al. 2017), implying no subcellular damage. That said, only one key component needs to lose function for the entire organism to become compromised. For example, the activity of myosin ATPase, a key enzyme for organismal muscle function, closely resembled whole-organism thermal performance curves and denatured at relatively low temperatures in eight lizard species corresponding closely to their respective CT_{MAX} (20% denatured between 37 and 45.2 °C; Licht 1964). Subcellular components such as ATPase could underlie thermal tolerance even though most components maintain function to higher temperature.

Given the complexities of subcellular interactions and the paucity of data for the thermal performance of subcellular components in reptiles and amphibians, heat shock protein (HSP) production might better indicate whether subcellular-level components are challenged at sub-critical high temperatures. HSPs commonly act as molecular chaperones, maintaining protein

structure and preventing aggregations of denatured proteins, and inducible variants are produced in response to cellular stress or damage (Fernando and Heikkila 2000; Kregel 2002; Daugaard et al. 2007). In diverse reptiles and amphibians, HSPs (particularly HSPA family members) are produced in response to sub-critical high temperatures, and production of HSPs can allow acclimation with increased thermal tolerance (Ulmasov et al. 1992; Fernando and Heikkila 2000; Zatespina et al. 2000; McMillan et al. 2011; Gao et al. 2014; Simoniello et al. 2016; Tedeschi et al. 2016). Moreover, patterns of HSP expression are correlated with thermal tolerance in lizards: warm-adapted species display higher constitutive HSP concentrations, and both initiate and maintain synthesis of HSPs to higher temperatures than more cold-adapted species (Ulmasov et al. 1992; Zatespina et al. 2000). This pattern of increased HSP production correlating with increases in thermal tolerance in reptiles and amphibians, along with observations for myosin ATPase in lizards, provides compelling evidence for subcellular-function loss playing a role in setting thermal limits, despite other subcellular components displaying little loss of function at relevant temperatures.

Organ-system mechanisms

Even though subcellular-level traits will be compromised at sufficiently high temperatures, higher-order systems might break down at lower temperatures and thus be proximally responsible for setting thermal tolerances (Pörtner 2001; 2002; Storch et al. 2014, but see Clark et al. 2013). The OCLTT hypothesis proposes that the highest organizational level in animals is the integrated cardiovascular and respiratory system because all tissues will be limited by their ability to acquire oxygen for respiration, and that this system is compromised by high temperatures prior to other systems (Pörtner 2001; 2002; Storch et al. 2014). However, similar to

other terrestrial species (Klok et al. 2004; McCue and De Los Santos 2013; Verberk et al. 2016) and many fish (Clark et al. 2013; Gräns et al. 2014; Norin et al. 2014; Wang et al. 2014; Ern et al. 2016), evidence for the OCLTT mechanism underlying thermal tolerance in reptiles and amphibians is limited. Under the OCLTT hypothesis, maximal and resting rates of oxygen consumption are expected to converge as animals reach their physiological limits at high temperatures, thereby reducing aerobic scope and potentially inducing a short-term reliance on anaerobic respiration (Frederich and Pörtner 2000; Pörtner and Knust 2007; Elaison et al. 2011; Verberk et al. 2013; Verberk et al. 2016; Table 1). Some evidence points to such a mechanism playing an important role in early animal evolution, notably in the transition to air breathing (Berner et al. 2007; Giomi et al. 2014; Teague et al. 2017). However, the few studies exposing animals to high temperatures and measuring indicators of aerobic and anaerobic respiration fail to find evidence for oxygen limitation in adult reptiles and amphibians (Carey 1979; Overgaard et al. 2012; Fobian et al. 2014; Gangloff et al. 2016; Telemeco et al. 2017). For example, oxygen consumption (\dot{V}_{O_2}) by pythons (*Python regius*) did not plateau at temperatures approaching CT_{MAX} either when at rest or during periods of high metabolic demand (Fobian et al. 2014, Fig. 1), and resting oxygen consumption in garter snakes (*Thamnophis elegans*) increased with temperature with no apparent limit when animals experienced near-lethal temperatures (Gangloff et al. 2016). Moreover, neither garter snakes (*T. elegans*) nor alligator lizards (*E. coerulea* and *E. multicarinata*) transitioned to anaerobic respiration when exposed to near-critical temperatures (Gangloff et al. 2016; Telemeco et al. 2017), despite snakes and lizards rapidly transitioning when oxygen availability is limited during exercise (reviewed in Gleeson 1991). Observations in amphibians are similar to those for reptiles. For example, oxygen consumption, arterial oxygen saturation, and the proportion of saturated hemoglobin did not plateau at high temperatures in

active or resting cane toads (*Rhinella marina*), thus providing evidence for these toads' ability to maintain a positive aerobic power budget at near-critical temperatures (Seebacher and Franklin 2011; Overgaard et al. 2012; Winwood-Smith et al. 2015). In both the boreal toad (*Anaxyrus boreas*) and leopard frog (*Lithobates pipiens*), aerobic scope increased with temperature to 30°C (Carey 1979). Whole-organism lactate concentration also increases with temperature, but there is no indication that either species becomes oxygen limited up to at least 30°C (Carey 1979). These results are in line with previous work showing that amphibians can maintain substantial aerobic scope at temperatures above active and preferred temperatures, although not necessarily at temperatures approaching T_{LETHAL} (Whitford 1973). While such studies provide strong evidence for adult reptiles and amphibians maintaining aerobic scope at high temperatures, we currently lack data on tissue and cellular oxygen supply, such as venous P_{O_2} , which would more directly test the mechanisms described by the OCLTT hypothesis (Pörtner et al. 2017; 2018).

A second approach for testing the influence of oxygen capacity on thermal constraints is to manipulate oxygen availability and examine changes in thermal behavior or tolerance (Table 1). Under the OCLTT hypothesis, hypoxia is predicted to reduce thermal optima and tolerance limits (Smith et al. 2015; Verberk et al. 2016; DuBois et al. 2017), and this prediction is somewhat supported in reptiles and amphibians. For example, CT_{MAX} , preferred body temperature (PBT), panting temperature (T_{PANT}), and gaping temperature (T_{GAPE}) are reduced when diverse species are exposed to very-low oxygen environments (< 10 kPa; mostly lizards examined; Hicks and Wood 1985; Dupre et al. 1986; Branco et al. 1993; Cadena and Tattersall 2009; Shea et al. 2016; DuBois et al. 2017; Fig. 2) and when hematocrit is experimentally reduced (only PBT examined; Hicks and Wood 1985; Wood 1990). Hypoxia-induced PBT reduction is a well-described phenomenon in ectotherms, termed “behavioral anapyrexia,” that

allows adaptive reduction of metabolic demand when oxygen is limited (Hicks and Wood 1985; Wood and Gonzales 1996; Steiner and Branco 2002; Hicks and Wang 2004), supporting the hypothesis that oxygen limitation influences thermal preference and possibly tolerance. Along with CT_{MAX} , reductions in T_{GAPE} and T_{PANT} , which provide an indication of perceived heat stress (Heatwole et al. 1973; Tattersall et al. 2006; DuBois et al. 2017), imply reduced thermal tolerance under hypoxia. Hypoxia also induces elevated heart rates and reduces both resting and active oxygen consumption (\dot{V}_{O_2}) in diverse species (Fig. 3), suggesting observed shifts in thermal tolerance and behavior are related to physiological limits of oxygen capacity (including diffusion and transport). Interestingly, only extreme hypoxia had the predicted effects on thermal tolerance and behavior, with levels of hypoxia within the range generally found in terrestrial environments having no effect (Figs. 2 and 3). Thus, it is not clear that the OCLTT mechanism will be generally relevant in nature.

In contrast to adult stages, naturalistic hypoxia reduces thermal performance and tolerance in larvae and eggs of reptiles and amphibians. Because amphibian eggs and larvae inhabit aqueous environments, environmental oxygen availability is reduced relative to terrestrial stages, thereby increasing the potential importance of the OCLTT mechanism similar to some fully aquatic ectotherms (e.g. Pörtner and Knust 2007; Verberk et al. 2013; Pörtner and Gutt 2016). Oxygen limitation is further exacerbated when species develop in small water bodies that can rapidly lose dissolved oxygen, such as ephemeral pools (Seymour and Bradford 1995; Sacerdote and King 2009), and because amphibian eggs are commonly produced within large gelatinous masses with low oxygen diffusion potential (Pinder and Friet 1994; Woods 1999; Sacerdote and King 2009). Although data are limited, these constraints appear to affect early stage amphibians congruent with oxygen limiting thermal tolerances. For example, amphibians

that nest in warm water produce smaller eggs and egg masses to facilitate oxygen diffusion to embryos (Woods 1999; Sacerdote and King 2009), and aquatic larvae born into warm water (from aquatic or terrestrial eggs) are smaller and have lower metabolic demands (Kuramoto 1975; Rollinson and Rowe 2018). In oviparous reptiles, embryonic gas exchange occurs via passive diffusion across the shell into the chorioallantoic membrane, which is much less efficient than adult respiration (Vitt and Caldwell 2009). As predicted by the OCLTT hypothesis, thermal tolerance is reduced when developing embryos experience modest to extreme hypoxia (Flewelling and Parker 2015; Liang et al. 2015; Smith et al. 2015). For example, lower environmental P_{O_2} experienced at high elevations reduces T_{LETHAL} in plateau fence lizard (*Sceloporus tristichus*) embryos compared to P_{O_2} at sea level (Smith et al. 2015), although such modest hypoxia does not affect the thermal tolerance of adult congeners (*S. occidentalis* and *S. graciosus*; Shea et al. 2016; DuBois et al. 2017). Thus, available data suggest that reptiles and amphibians experience ontogenetic shifts in the proximate mechanism underlying thermal tolerance: early stages appear more affected by organ-system-level failures, such as the OCLTT mechanism, whereas later stages are more impacted by subcellular mechanisms.

An integrative framework: Hierarchical Mechanisms of Thermal Limitation

Available evidence implies that neither failure of subcellular components nor oxygen and capacity limitation are solely responsible for loss of performance at high temperatures in reptiles and amphibians. Rather, mechanisms across levels of organization appear to play partial, context-dependent roles. We propose a unified framework that combines subcellular mechanisms, the oxygen and capacity limited thermal tolerance (OCLTT) hypothesis, and the

thermal performance curve (TPC) paradigm. We call this integrative framework “Hierarchical Mechanisms of Thermal Limitation” (HMTL, Fig. 1). Like the OCLTT hypothesis, we propose that oxygen diffusion and transport capacity largely shape thermal performance at sub-critical temperatures via effects of temperature on aerobic scope. However, the HMTL framework explicitly recognizes the importance of subcellular-level mechanisms and allows them to underlie absolute thermal limits, such as CT_{MAX} and T_{LETHAL} . Under this hypothesis, the relative importance of subcellular- and organ-system-level mechanisms on thermal tolerance is context dependent, but predictable. We propose that the HMTL framework explains diverse empirical results and can bring together decades of physiological studies in reptiles and amphibians.

Given that numerous models of thermal performance and tolerance have been proposed, we think that it is useful to state their similarities and differences with HMTL. The HMTL framework builds upon ideas developed for the OCLTT hypothesis proposed by Pörtner and colleagues (recently reviewed in Pörtner et al. 2017) and is similar to that put forth by Ern et al. (2016) for fishes (see Fig. 1 in Ern et al. 2016), but more formally incorporates the TPC paradigm and explicitly describes mechanism-dependent critical temperatures. Importantly, this hypothesis predicts conditions under which subcellular and organ-system failure will set thermal tolerance limits and when transitions are likely to occur. Our framework also differs importantly from the Multiple Performances – Multiple Optima (MPMO) framework put forth by Clark et al. (2013) for fishes. The MPMO suggests a single thermal limit set by an undefined mechanism but multiple, trait-specific optima that can dramatically differ from the temperature that maximizes aerobic scope (summarized in Fig. 7 of Clark et al. 2013). By contrast, we propose multiple potential mechanisms of thermal limitation but a single whole-organism thermal optimum for

performance correlated with the temperature that maximizes aerobic scope (although this will be an integration across traits).

Figure 1 illustrates the HMTL framework. In the top row of panels, solid blue and red lines represent the effect of temperature on oxygen consumption during rest and activity, respectively. Assuming activity is maximal, aerobic scope is the difference between these lines. To illustrate this concept with empirical data, we derived these lines by fitting a generalized logistic function to data from Fobian et al. (2014) for pythons (*Python regius*) assuming the shape of the response curve is the same during rest and activity. Based on available data, the general shape of these curves is qualitatively similar across diverse taxa (e.g. Fobian et al 2014, Overgaard 2012, Carey 1979, Frederich 2000, Ern et al. 2016) and we think that the predictions of the HMTL framework can be generalized beyond the specific data used to generate this illustration. The dotted blue and red lines depict exponential functions fit to these data and illustrate predicted oxygen demand/use if organisms were unconstrained by capacity limitations. The columns illustrate predicted effects of variable oxygen environments, and the bottom row displays TPCs predicted to result given relationships in the top row.

A central assumption of the HMTL framework is that critical thermal limits exist for both subcellular mechanisms (subcellular T_{CRIT}) and aerobic respiration (aerobic T_{CRIT}), and that organismal thermal limits are proximally caused by the lower T_{CRIT} (Fig. 1). The environment and oxygen-handling capacity of the organism will co-determine the hierarchy of these critical limits and thus their relative importance. The subcellular T_{CRIT} is the temperature at which key subcellular components begin to lose function, and should be insensitive to activity state or oxygen environment. By contrast, aerobic T_{CRIT} is the temperature at which oxygen capacity is maximized, and is affected by activity state and oxygen environment. For example, aerobic T_{CRIT}

during rest will be higher than aerobic T_{CRIT} during activity because elevated O_2 demand during activity causes capacity limits to be reached more readily (Fig 1). Moreover, both active- and resting-aerobic T_{CRIT} should be reduced during exposure to hypoxic environments (Figs. 1B-1C). Any reduction in environmental oxygen availability below normoxia will reduce aerobic scope. However, CT_{MAX} will only be reduced if environmental hypoxia is sufficient to cause resting-aerobic T_{CRIT} to drop below subcellular T_{CRIT} (Fig. 1C). The environmental oxygen tension where resting-aerobic T_{CRIT} equals subcellular T_{CRIT} is P_{CTMAX} as defined by Ern et al. (2016).

By integrating T_{CRIT} values and aerobic scope, we can derive whole-organism TPCs (Fig. 1, bottom row). Because the active-aerobic T_{CRIT} is the lowest temperature where maximal metabolic rate can be achieved, it closely corresponds to the optimal temperature for aerobic performance (whole-organism T_{OPT}) where aerobic scope is maximized. At temperatures below active-aerobic T_{CRIT} , we predict that performance increases with temperature proportional to aerobic scope. At temperatures above active-aerobic T_{CRIT} , performance will drop until CT_{MAX} is reached, but we predict that the shape of this drop will depend on which T_{CRIT} underlies CT_{MAX} . If resting-aerobic T_{CRIT} underlies CT_{MAX} , the TPC should be more symmetrical with performance and aerobic scope decreasing at a rate mirroring the increase (Fig 1C). However, if subcellular T_{CRIT} underlies CT_{MAX} , the curve will be asymmetric with loss of performance occurring more rapidly the closer active-aerobic T_{CRIT} is to subcellular T_{CRIT} (Figs. 1A-1B).

Assumptions, predictions, and evidence for Hierarchical Mechanisms of Thermal Limitation in reptiles and amphibians

The HMTL framework produces numerous testable predictions, some of which can be addressed with available data, while others offer exciting avenues for future research. First, any

environmental or organismal characteristics that reduce oxygen availability or capacity for oxygen utilization (e.g. aquatic respiration, reliance on cutaneous gas exchange, or life-stages such as eggs with reduced diffusion potential) should increase the probability of resting-aerobic T_{CRIT} underlying CT_{MAX} . This can explain oxygen capacity setting thermal limits in embryos (Smith et al. 2015) but not adults (Overgaard et al. 2012; Fobian et al. 2014; DuBois et al. 2017). Moreover, the transition between aerobic and subcellular T_{CRIT} predicted by HMTL explains extreme experimental hypoxia reducing thermal tolerance in adult reptiles and amphibians, while moderate hypoxia does not (Fig. 1C and Figs. 2B–2D). Similarly, HMTL predicts that CT_{MAX} is lower when governed by oxygen limitation than when governed by subcellular mechanisms. Supporting this prediction, CT_{MAX} drops in tadpoles as oxygen demand increases with growth and when respiratory structures are compromised in late-stage metamorphs, but frequently elevates again in terrestrial adults presumably as a result of increased oxygen capacity (ShermanCupp 1980; Sherman 1980; Floyd 1983).

Another important prediction of the HMTL hypothesis is that performance can be limited by oxygen exchange capacity at high temperatures even when CT_{MAX} and T_{LETHAL} are proximally set by subcellular mechanisms. Some interpretations of the OCLTT hypothesis similarly highlight limits on performance at *pejus* rather than critical temperatures (Pörtner and Knust 2007; Pörtner 2014; Verberk et al. 2016; Pörtner et al. 2017), but other workers suggest OCLTT should explain critical limits to be useful (i.e. Fobian et al. 2014; Smith et al. 2015; Ern et al. 2016; Verberk et al. 2016; DuBois et al. 2017). Under the HMTL framework, activity state is assumed to have no appreciable effect on oxygen capacity, although this may not hold in some highly aerobic taxa (e.g. Wang and Hicks 2004). The extent to which activity-induced increases in oxygen capacity affect thermal performance curves and limits is an important direction for

future work. Generally however, individuals in metabolically-demanding states are expected to reach capacity limits at temperatures below those at which such limits are reached by resting individuals. Anaerobic respiration might compensate for short-term mismatches between energy demand and oxygen capacity (reviewed in Gleeson 1991; Fig. 1A, red shaded region), but aerobic constraints are predicted to reduce performance during long-term activity (e.g. digestion, reproduction, recovery; Jackson 2007; Pörtner et al. 2017).

In diverse species, active metabolic rate asymptotes despite increases in cardiovascular output, implying oxygen flux becomes limited at temperatures above active-aerobic T_{CRIT} (e.g. Bartholomew and Tucker 1963; Bennett and Licht 1972; Wilson 1974; Overgaard et al. 2012; Fobian et al. 2014). Furthermore, pulmonary diffusion capacity is limited by reduced plasma gas solubility and hemoglobin binding affinity at high temperatures (Wood and Moberly 1970; Kinney et al. 1977; Pough 1980; Jackson 2007; da Silva et al. 2013). Diffusion limitation of cutaneous gas exchange is also well established (reviewed in Burggren 1988; Wang 2011) and will be important in species that spend considerable time submerged (e.g. Ultsch 1973) or are lungless (e.g. Plethodontid salamanders; Whitford and Hutchison 1965; Spotilla 1972). Finally, increases in the products of anaerobic respiration, such as lactic acid, can lead to blood acidification and thereby further declines in blood oxygen affinity (Bennett 1973). The interaction of numerous factors at high temperatures reduces oxygen capacity, which in turn limits aerobic performance at high temperatures in reptiles and amphibians, regardless of the mechanism governing CT_{MAX} (further reviewed in Jackson 2007).

The HMTL framework predicts that whole-organism T_{OPT} is the temperature providing maximal aerobic scope, which is governed by active-aerobic T_{CRIT} . Thus, alleviating the limits that underlie active-aerobic T_{CRIT} should allow increased maximal performance (i.e. aerobic

scope) and T_{OPT} (Fig. 1). Unfortunately, few data are available comparing whole-organism T_{OPT} and aerobic scope in reptiles or amphibians. In the snake *P. regius*, whole-organism T_{OPT} and the temperature where aerobic scope is maximized appear to correspond (Fobian et al. 2014). In the toad *R. marina*, observations are more complex and suggest that the maintenance of aerobic scope to high temperatures could be independent of whole-organism T_{OPT} , at least in some cases. Hopping performance is maximized at $\sim 30^{\circ}\text{C}$ (Kearney et al. 2008) whereas aerobic scope can plateau at 30°C , but can also increase to at least 40°C depending on acclimation treatment (Overgaard et al. 2012). Additional data are needed to determine if acclimation elevates T_{OPT} for whole-organism performance similar to maximal aerobic scope in *R. marina*, as would be predicted by the HMTL framework.

Observations for PBT provide additional indirect evidence that whole-organism T_{OPT} corresponds to the temperature that maximizes aerobic scope in reptiles, but again data for amphibians are less clear. Generally, terrestrial ectotherms thermoregulate to within a narrow thermal range during activity if costs to thermoregulation are not prohibitively high (Huey 1982; Bauwens et al. 1995; Angilletta 2009; Kingsolver and Buckley 2015; Sears et al. 2016). Natural selection is predicted to shape thermal preference such that PBT corresponds to, or is slightly below, whole-organism T_{OPT} (Huey 1982; Bauwens et al. 1995; Angilletta et al. 2002; but see Huey and Bennett 1987). As predicted, PBT and temperature of maximum aerobic scope are highly concordant in lizards exposed to normoxic environments (Wilson 1974). On the other hand, in the boreal toad (*Anaxyrus boreas*), aerobic scope is maximal at 30°C whereas T_{PREF} is 24°C (Carey 1978; 1979). Interestingly, *A. boreas* and other anurans (*L. pipiens*, Carey 1979; *R. marina*, Overgaard et al. 2012) exhibit an increase in lactic acid production with temperatures above PBT in both resting and active animals. Increased lactic acid production indicates that

high temperatures induce anaerobic respiration, even as aerobic scope is maintained, and therefore incur an oxygen debt for recovery. Further data are needed to assess whether the potential mismatch between maximal aerobic scope and T_{OPT} in anurans can be explained by animals balancing the increased costs of repaying oxygen debt resulting from increased anaerobic respiration at high temperatures with the benefits of concurrent increases in aerobic scope.

Assuming maximal aerobic scope, T_{OPT} , and PBT are linked as predicted by HMTL, they are not uniformly affected by experimental hypoxia as might initially be predicted. Only extreme hypoxia affects PBT (Fig. 2) whereas the temperature that maximizes aerobic scope and presumably whole-organism T_{OPT} is predicted to drop continuously with hypoxia (Fig. 1). This discrepancy might indicate that adult reptiles and amphibians cannot sense and respond to hypoxia-induced changes in aerobic scope in real time, which might be expected given that these animals evolved in terrestrial environments where oxygen availability is relatively stable within a lifetime. Thus, we propose that individuals choose the same body temperature regardless of the oxygen environment so long as basic metabolic demands are met, and thus predict that the “breakpoint” in Fig. 2A occurs when hypoxia causes resting-aerobic T_{CRIT} to fall below evolved PBT. Data comparing resting aerobic T_{CRIT} and PBT when oxygen environment or demand is manipulated are needed to test this prediction. Given the predicted relationships between aerobic scope, T_{OPT} , and PBT, we also expect species adapted to low-oxygen environments to have relatively lower T_{OPT} and PBT, or greater oxygen-handling capacity. However, covariation between temperature and oxygen with elevation make testing this prediction in terrestrial environments difficult.

In addition to further exploring the potential importance of resting- and active-aerobic T_{CRIT} , additional research is needed to identify the subcellular components that underlie subcellular T_{CRIT} . A subset of evolutionarily conserved components might constrain thermal tolerance across a diversity of taxa, or the components that are most important could be taxonomically specific. Currently, the data needed to differentiate these possibilities are not available. We think that measures of ATPase and HSP provide useful candidates for further exploration, but caution that focusing on a single or few potential indicators in isolation will likely provide a contorted view of subcellular limitation. Advances in “-omics” technologies, particularly differential expression RNAseq, metabolomics, and proteomics could provide much useful information about subcellular physiological function, and provide additional candidate molecules for detailed analysis (e.g. Verberk et al. 2013; Williams et al. 2014; Campbell-Staton et al. 2017; Telemeco et al. 2017). We recommend that experiments manipulating the thermal and oxygen environment of organisms endeavor to collect subcellular data as well as whole-organism performance data. Where possible, an integrative approach combining measurements of subcellular components and whole-organism performance will best illuminate the mechanisms that underlie tolerance and their interactions.

Finally, the HMTL framework makes predictions for how populations could be affected by global change. Numerous species are expanding or shifting their range to higher elevations in response to climate change-related temperature increases (e.g. Sinervo et al. 2010; Pincheira-Donoso et al. 2013; Pauchard et al. 2016). However, HMTL predicts that reduced oxygen partial pressures at high elevation will lower both T_{OPT} and maximal performance. Thus, species must seek cooler environments as they move to higher elevation to maintain optimal performance, and performance potential will go down regardless of thermal environment selected. An evolutionary

change appears necessary for animals to seek out reduced body temperatures because moderate hypoxia does not affect PBT (Fig. 2). The HMTL framework also predicts that species with greater oxygen capacity will be more buffered from lost performance when exposed to increased environmental temperatures. Thus, oxygen capacity might be a prime target of natural selection as climates warm, even if it does not underlie CT_{MAX} or T_{LETHAL} . Finally, given the great diversity in modes of gas exchange, metabolic demands, and shifts across life-history stages in reptiles and amphibians, we emphasize the need to explore these hypotheses in a greater number and variety of taxa.

Conclusions

Available data suggest that both subcellular- and organ-system-level mechanisms shape thermal performance and tolerance in amphibians and reptiles. The HMTL framework that we propose describes how both mechanisms co-affect animals, with their relative importance driven by their respective T_{CRIT} . We think that the HMTL hypothesis improves upon current frameworks by explicitly removing the false dichotomy between subcellular mechanisms and oxygen limitation, identifying useful parameters for further research (subcellular T_{CRIT} , resting-aerobic T_{CRIT} , and active-aerobic T_{CRIT}), and describing how aerobic and subcellular limitations interact to affect TPCs. Moreover, HMTL appears to explain a wide range of initially perplexing observations in reptiles and amphibians such as reduced aerobic scope at high temperatures without aerobic failure at critical temperatures, extreme hypoxia affecting thermal tolerance and behavior in adults with no effect of moderate hypoxia, moderate hypoxia reducing thermal tolerance in embryos, and HSP and ATPase activity suggesting loss of subcellular function near critical temperatures. Still, the HMTL framework makes numerous predictions for which additional data

are needed, including animals only reducing their body temperature when resting-aerobic T_{CRIT} drops below PBT, CT_{MAX} being lower when resting-aerobic T_{CRIT} is responsible than when subcellular T_{CRIT} is responsible, maximum aerobic capacity underlying whole-organism thermal optima, and reduced performance when species invade higher elevations without increased oxygen capacity. Moreover, virtually no data are available to address the potential importance of aerobic- or subcellular- T_{CRIT} as evolutionary constraints shaping the adaptive landscape. Further data better representing the diversity of reptile and amphibian taxa are needed both to understand the potential relevance of the HMTL mechanism in extant reptiles and amphibians, and how such a mechanism could have shaped the evolution of these animals. We think that many of the ideas that make up our HMTL framework are already widely accepted within the scientific community, and hope that explicitly describing them within a single framework with clear, testable predictions will facilitate further research. Although inspired by reptiles and amphibians, this integrated framework could have broad applicability across ectothermic animals. We look forward to continued investigation further integrating, refining, and testing these ideas across mechanisms and taxa.

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Supplementary data

Summary data compiled from the literature for Figs 2-3 are available in Table S1 and available at *ICB* online.

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Figure legends

Fig. 1 Schematic of the Hierarchical Mechanisms of Thermal Limitation (HMTL) framework illustrating proposed relationships between body temperature, oxygen environment, metabolism, thermal limits, and performance. The top row of plots displays resting and active metabolic rates as a function of temperature within thermal and physiological limits, and the bottom row displays predicted thermal performance curves. Panel A) displays these relationships in normoxic environments. Data are means \pm s.e.m. for the snake, *Python regius*, from Fobian et al. (2014). These data were used to fit curves for resting and active metabolic rate, which were then used to estimate T_{OPT} (maximal aerobic scope), resting-aerobic T_{CRIT} , active-aerobic T_{CRIT} , and thermal performance curve shape. The critical thermal maximum is 42°C (Fobian et al. 2014). Panel B) Moderate hypoxia (10-20 kPa) is predicted to have no effect on CT_{MAX} or resting metabolism because resting aerobic T_{CRIT} is above subcellular T_{CRIT} . Even so, active-aerobic T_{CRIT} will be reduced thereby lowering aerobic scope, maximal performance, and T_{OPT} . Panel C), Exposure to extreme hypoxia (< 10 kPa) will reduce CT_{MAX} because resting-aerobic T_{CRIT} drops below subcellular T_{CRIT} . Active-aerobic T_{CRIT} will be strongly affected by such hypoxic environments as well, with large reductions in aerobic scope, maximal performance, and T_{OPT} . In Panels B) and C), gray lines illustrate values predicted for normoxia and are included to aid interpretation of changes predicted to result from reduced environmental oxygen availability. Similarly, arrows in Panels B) and C) indicate predicted displacement of maximum oxygen capacity, resting-aerobic T_{CRIT} , T_{OPT} , and CT_{MAX} values given the oxygen environment. Subcellular T_{CRIT} is not predicted to be affected by oxygen environment and therefore is never displaced.

Fig. 2 Thermal preference and tolerance are unaffected by moderate hypoxia but reduced in extremely hypoxic environments (< 10 kPa). This “broken-stick” pattern is apparent for A) preferred body temperature (i.e. behavioral anapyrexia), B) critical thermal maximum, C) gaping temperature, and D) panting temperature across species. Data are mostly available for lizards (solid lines) but preferred body temperature data are also available for alligator (dashed line). Lines between points are included to aid visualization, but connections between values at normoxia and hypoxia are likely non-linear. Colors (online only) and initials denote species: A.m. = *Alligator mississippiensis*, B.v. = *Basiliscus vittatus*, C.p. = *Ctenosaura pectinata*, I.i. = *Iguana iguana*, P.v. = *Pogona vitticeps*, S.g. = *Sceloporus graciosus*, S.o. = *Sceloporus occidentalis*, V.e. = *Varanus exanthematicus*, V.v. = *Varanus varius*. Data are means \pm s.e.m. derived from the literature. See Table S1 for data and citations.

Fig. 3 As the environment becomes increasingly hypoxic, A) arterial P_{O_2} decreases, whereas B) resting metabolic rate, C) heart rate, and D) maximal metabolic rate are relatively unaffected until the environment becomes extremely hypoxic (<10 kPa), similar to thermal preference and tolerance (see Fig. 2 for comparison). Colors (online only) denote taxonomic group whereas initials denote species: A.c. = *Anaxyrus cognatus* (formerly *Bufo cognatus*), A.m. = *Alligator mississippiensis*, C.s. = *Chelydra serpentina*, D.d = *Dipsosaurus dorsalis*, L.p. = *Lithobates pipiens* (formerly *Rana pipiens*), P.m. = *Pituophis melanoleucus*, R.m. = *Rhinella marina* (formerly *Bufo marinus*), R.s. = *Rhinella schneideri* (formerly *Bufo paracnemis*). A.c. L.p. R.s. and R.m. are frogs, D.d. is a lizard, P.m. is a snake, C.s. is a turtle, and A.m. is an alligator. Experimental temperature is given to the right of the species’ initials. Data are means \pm s.e.m. derived from the literature. See Table S1 for data and citations.

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Box 1 List of terms and abbreviations

ATP	Adenosine triphosphate
active-aerobic T_{CRIT}	Critical temperature where aerobic respiration is maximized for active individuals (i.e. $\dot{V}_{\text{O}_2\text{MAX}}$ is maximized, °C)
resting-aerobic T_{CRIT}	Critical temperature where aerobic respiration would be maximized for resting individuals, assuming they survive to such high temperatures (i.e. $\dot{V}_{\text{O}_2\text{REST}}$ is maximized, °C)
subcellular T_{CRIT}	Critical temperature for subcellular function (°C)
CT_{MAX}	Critical thermal maximum (°C)
HMTL	Hierarchical Mechanisms of Thermal Limitation hypothesis
HSP	Heat shock protein
MPMO	Multiple Performances – Multiple Optima hypothesis (sensu Clark et al. 2013)
OCLTT	Oxygen- and Capacity-Limited Thermal Tolerance hypothesis (sensu Pörtner 2002 and Pörtner et al. 2017)
T_{GAPE}	Gaping temperature: Temperature at which animals gape to promote evaporative cooling (°C)
T_{LETHAL}	Lethal temperature: Temperature at which an organism dies under acute exposure (°C)
T_{OPT}	Optimal temperature for performance (°C)
T_{PANT}	Panting temperature: Temperature at which animals pant to promote evaporative cooling (°C)
T_{PEJUS}	Pejus (i.e. getting worse) temperature (°C, sensu Frederich and Pörtner 2000)
TPC	Thermal performance curve (sensu Huey and Stevenson 1979)
PBT	Preferred body temperature (°C)
P_{CTMAX}	Oxygen partial pressure below which CT_{MAX} is reduced (kPa) (sensu Ern et al. 2016)
P_{O_2}	Partial pressure of oxygen (kPa)
P_{CO_2}	Partial pressure of carbon dioxide (kPa)
\dot{V}_{O_2}	Oxygen consumption rate (generally mL O ₂ min ⁻¹)

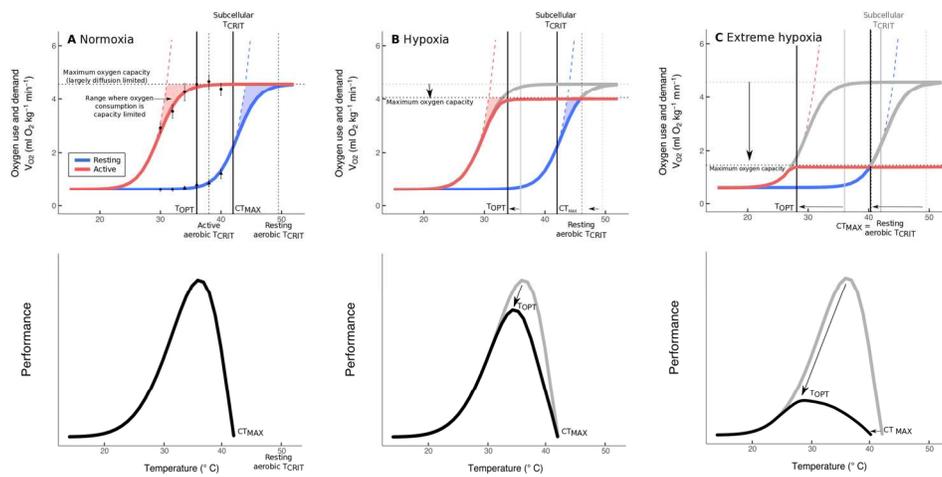
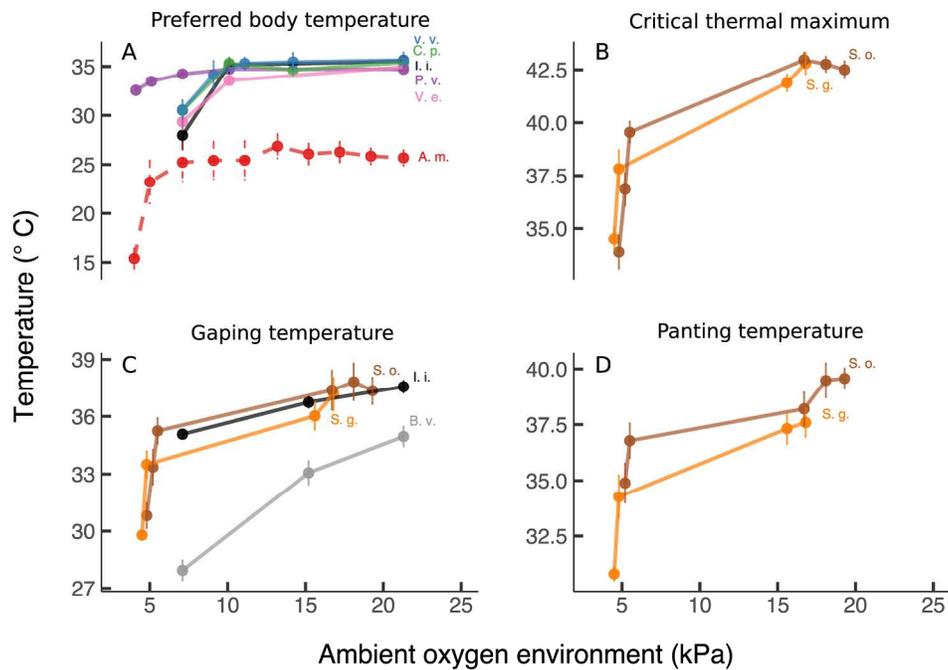


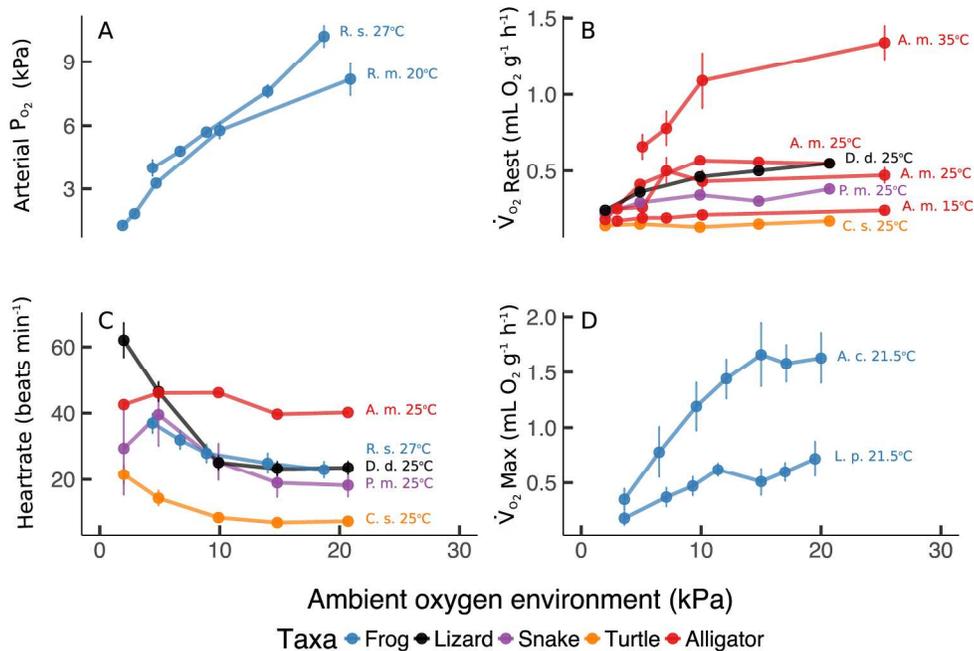
Fig. 1 Schematic of the Hierarchical Mechanisms of Thermal Limitation (HMTL) framework illustrating proposed relationships between body temperature, oxygen environment, metabolism, thermal limits, and performance. The top row of plots displays resting and active metabolic rates as a function of temperature within thermal and physiological limits, and the bottom row displays predicted thermal performance curves. Panel A) displays these relationships in normoxic environments. Data are means \pm s.e.m. for the snake, *Python regius*, from Fobian et al. (2014). These data were used to fit curves for resting and active metabolic rate, which were then used to estimate TOPT (maximal aerobic scope), resting-aerobic TCRT, active-aerobic TCRT, and thermal performance curve shape. The critical thermal maximum is 42°C (Fobian et al. 2014). Panel B) Moderate hypoxia (10-20 kPa) is predicted to have no effect on CTMAX or resting metabolism because resting aerobic TCRT is above subcellular TCRT. Even so, active-aerobic TCRT will be reduced thereby lowering aerobic scope, maximal performance, and TOPT. Panel C), Exposure to extreme hypoxia (< 10 kPa) will reduce CTMAX because resting-aerobic TCRT drops below subcellular TCRT. Active-aerobic TCRT will be strongly affected by such hypoxic environments as well, with large reductions in aerobic scope, maximal performance, and TOPT. In Panels B) and C), gray lines illustrate values predicted for normoxia and are included to aid interpretation of changes predicted to result from reduced environmental oxygen availability. Similarly, arrows in Panels B) and C) indicate predicted displacement of maximum oxygen capacity, resting-aerobic TCRT, TOPT, and CTMAX values given the oxygen environment. Subcellular TCRT is not predicted to be affected by oxygen environment and therefore is never displaced.

176x88mm (300 x 300 DPI)



Thermal preference and tolerance are unaffected by moderate hypoxia but reduced in extremely hypoxic environments (< 10 kPa). This "broken-stick" pattern is apparent for A) preferred body temperature (i.e. behavioral anapyrexia), B) critical thermal maximum, C) gaping temperature, and D) panting temperature across species. Data are mostly available for lizards (solid lines) but preferred body temperature data are also available for alligator (dashed line). Lines between points are included to aid visualization, but connections between values at normoxia and hypoxia are likely non-linear. Colors (online only) and initials denote species: A.m. = Alligator mississippiensis, B.v. = Basiliscus vittatus, C.p. = Ctenosaura pectinata, I.i. = Iguana iguana, P.v. = Pogona vitticeps, S.g. = Sceloporus graciosus, S.o. = Sceloporus occidentalis, V.e. = Varanus exanthematicus, V.v. = Varanus varius. Data are means \pm s.e.m. derived from the literature. See Table S1 for data and citations.

231x168mm (300 x 300 DPI)



As the environment becomes increasingly hypoxic, A) arterial P_{O_2} decreases, whereas B) resting metabolic rate, C) heart rate, and D) maximal metabolic rate are relatively unaffected until the environment becomes extremely hypoxic (<10 kPa), similar to thermal preference and tolerance (see Fig. 2 for comparison).

Colors (online only) denote taxonomic group whereas initials denote species: A.c. = *Anaxyrus cognatus* (formerly *Bufo cognatus*), A.m. = *Alligator mississippiensis*, C.s. = *Chelydra serpentina*, D.d. = *Dipsosaurus dorsalis*, L.p. = *Lithobates pipiens* (formerly *Rana pipiens*), P.m. = *Pituophis melanoleucus*, R.m. = *Rhinella marina* (formerly *Bufo marinus*), R.s. = *Rhinella schneideri* (formerly *Bufo paracnemis*). A.c. L.p. R.s. and R.m. are frogs, D.d. is a lizard, P.m. is a snake, C.s. is a turtle, and A.m. is an alligator. Experimental temperature is given to the right of the species' initials. Data are means \pm s.e.m. derived from the literature. See Table S1 for data and citations.

222x159mm (300 x 300 DPI)

Table 1. Experimental designs for testing aspects of the Hierarchical Mechanisms of Thermal Limitation hypothesis in reptiles and amphibians with example studies.

Experiment type: Factor manipulated	Manipulation	Dependent variable	What it demonstrates	Examples
Temperature	Temperature treatments or ramp in lab	Oxygen capacity parameters (\dot{V}_{O_2} , Active \dot{V}_{O_2} , alveolar/arterial P_{O_2} , heart rate), lactate production	Maintenance of aerobic scope at high temperatures; No evidence of transition to anaerobic respiration	Carey 1979 Seebacher and Franklin 2011 Overgaard et al. 2012 Fobian et al. 2014 Gangloff et al. 2016
	Temperature treatments in lab	Skeletal muscle metabolome, mitochondrial function	No transition from aerobic to anaerobic metabolism or subcellular damage at high temperatures	Telemeco et al. 2017
	Ex vivo temperature treatments	Enzyme activity	Temperature where subcellular components lose function	Licht 1964 Paulson and Hutchinson 1987 Irie et al. 1998
	Temperature treatments in lab	Heat shock protein induction	Temperature that induces a subcellular-protection response	Al-Jassabi 2002 Ulmasov et al. 1992 Fernando and Heikkila 2000 Zatespina et al. 2000 McMillan et al. 2011 Gao et al. 2014 Simoniello et al. 2016 Tedeschi et al. 2016
Ambient Oxygen	Oxygen treatments in lab	Oxygen capacity parameters (\dot{V}_{O_2} , heartrate, alveolar/arterial P_{O_2} , ventilation rate)	Aerobic capacity is maintained under conditions of mild hypoxia, but is limited under extreme hypoxia	Boyer 1963 Boyer 1966 Withers and Hillman 1983 Pörtner et al. 1991 Branco et al. 1993 Wang et al. 1994
	Oxygen treatments in lab	PBT	PBT is unaffected by mild hypoxia, but is reduced under extreme hypoxia	Hicks and Wood 1985 Branco et al. 1993 Cadena and Tattersall 2009

Both Ambient Oxygen levels & Temperature	Temperature and oxygen treatments in lab	Embryo development and survival	Hyperoxia increases survivorship while hypoxia reduces survivorship at high temperatures; Hypoxia reduces development, growth, and hatchling performance at high temperatures	Flewelling and Parker 2015 Liang et al. 2015 Smith et al. 2015
	Temperature gradient and/or ramp and oxygen treatments in lab	CT_{MAX} , T_{GAPE} , T_{PANT}	Behavioral responses to high temperatures depend on ambient O_2 , but only under extreme hypoxia	Dupre et al. 1986 Tattersall and Gerlach 2005 Shea et al. 2016
	Transplant across altitudinal gradients within species' ranges; Manipulation of ambient O_2 in field; Temperature ramp	CT_{MAX} , T_{GAPE} , T_{PANT}	Behavioral responses to high temperatures depend on ambient O_2 , but only under extreme hypoxia	DuBois et al. 2017
Oxygen capacity	Hematocrit reduction	PBT	Reduced oxygen capacity affects temperature perception and animals choose lower temperatures	Wood 1990 Hicks and Wood 1985
	Blood volume reduction	Oxygen capacity parameters (heartrate, alveolar/arterial P_{O_2} , ventilation rate)	Heart rate increases while ventilation rate is unchanged by reduced oxygen carrying capacity	Wang et al. 1994
Observational	--	Quantification of CT_{MAX} across life stages	Oxygen capacity limits thermal tolerance at some developmental stages in larval anurans	Cupp 1980 Sherman 1980 Floyd 1983
	--	Comparison of PBT, resting \dot{V}_{O_2} , Active \dot{V}_{O_2} ,	PBT matches temperature of maximal aerobic scope in lizards	Wilson 1974