Aerobic scope and cardiovascular oxygen transport is not compromised at high temperatures in the toad *Rhinella marina*

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Running title: Aerobic Scope in *Rhinella marina*

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Abstract

Numerous recent studies convincingly correlate the upper thermal tolerance limit of aquatic ectothermic animals to reduced aerobic scope, and ascribe the decline in aerobic scope to failure of the cardiovascular system at high temperatures. In the present study we investigate whether this “aerobic scope model” applies to an air-breathing and semi-terrestrial vertebrate *Rhinella marina* (formerly *Bufo marinus*). To quantify aerobic scope, we measured resting and maximal rate of oxygen consumption at temperatures ranging from 10 to 40°C. To include potential effects of acclimation, three groups of toads were acclimated chronically at 20, 25 and 30°C, respectively. The absolute difference between resting and maximal rate of oxygen consumption increased progressively with temperature and there was no significant decrease in aerobic scope, even at temperature immediately below the lethal limit (41-42°C). Hematological and cardio-respiratory variables were measured at rest and immediately after maximal activity at benign (30°C) and critically high (40°C) temperatures. Within this temperature interval, both resting and active heart rate increased, and there was no indication of respiratory failure, judged from high arterial oxygen saturation, PO2 and [HbO2]. With the exception of elevated resting metabolic rate for cold acclimated toads, we found few differences in the thermal responses between acclimation groups with regard to the cardio-metabolic parameters. In conclusion, we found no evidence for temperature induced cardio-respiratory failure in *Rhinella marina* indicating that maintenance of aerobic scope and oxygen transport is unrelated to the upper thermal limit of this air-breathing semi-terrestrial vertebrate.

**Keywords:** Oxygen limitation hypothesis, amphibian, *Bufo marinus*, climate change, heat tolerance, pejus, maximal energy consumption, ventilation, heart rate
Introduction

Temperature directly affects the rates of virtually all physiological and biochemical processes, and environmental temperature is arguably one of the most important factors defining the fundamental niche for animals (Cossins and Bowler, 1987; Schmidt-Nielsen, 1997; Angilletta, 2009). Given the direct influence of temperature on population growth, survival and sustained motor function, it is not surprising that shifts in species distributions can be correlated to the current rise in global temperature (Parmesan et al., 2003; Thomas et al., 2004; Perry et al., 2005). To understand and model how future global warming scenarios will affect species, a predictive framework is needed, and different approaches are therefore being promoted to assess the relative susceptibility of species to climate change (Deutsch et al., 2008; Pörtner and Farrell, 2008; Williams et al., 2008; Somero, 2010; Hofmann and Todgham, 2010). Although still debated (e.g. Clark et al., 2005a; 2011; Marshall et al., 2011 Keen and Gamperl, 2012), an influential physiological approach emphasizes the negative effects of high temperature on aerobic scope, i.e. the decline in the difference between minimal oxygen requirements and maximal rate of oxygen uptake as temperature increases (Pörtner, 2001; Pörtner and Knust, 2007). Thus, a number of studies convincingly correlate reduced aerobic scope to environmental and climate-related impacts on performance and fitness (Pörtner and Knust, 2007; Eliason et al., 2011; Neuheimer et al., 2011). The theoretical rationale is that the aerobic scope determines the scope for fitness-related activities, such as feeding, locomotion, growth, and reproduction (Fry and Hart, 1948; McCormick et al., 1972; Pörtner, 2001). Most studies linking aerobic scope to whole animal performance in vertebrates, however, concern aquatic organisms. Thus, it has rarely been studied whether aerobic scope provides a reliable indicator of thermal sensitivity and fitness in air-breathing vertebrates.

The “aerobic scope model” predicts that parts of the oxygen transport cascade become limiting at high temperature. Air-breathing vertebrates differ from water-breathing vertebrates in at least two respects that could potentially influence the thermal sensitivity of the oxygen transport cascade. Firstly, because oxygen solubility in water decreases with increasing temperature, the ventilatory requirements of water-breathers is expected to increase proportionally more than metabolism when temperature increases (Dejours, 1975). Secondly, the cardiovascular systems of many air-breathing ectothermic vertebrates, including amphibians, are more complex than in fish (e.g. Johansen and Hanson, 1968; Wang et al., 1999). Thus, while the fish heart partially relies on oxygen of venous blood returning to
the heart, the left side of the ventricle in air-breathing ectothermic vertebrates receives
oxygenated blood returning from the lungs (MacKinnon and Heatwole, 1981; Farmer, 1997;
Farrell et al., 2009).

Due to the differences highlighted above, it is clear that ventilatory and cardiovascular
challenges at high temperature differ between air- and water-breathing ectothermic
vertebrates. The objective of the present study is to examine how aerobic scope changes with
temperature in an air-breathing amphibian *Rhinella marina*. Numerous studies show that
aerobic scope increases with temperature in amphibians, including toads (Whitford, 1973;
Carey, 1979), and the thermal dependence of the cardio-respiratory system has previously
been studied in *Rhinella marina* (Feder, 1982; Hillman et al., 1985; Hillman, 1987; Withers
et al., 1988; Gamperl et al., 1999; Hedrick et al., 1999; Andersen et al., 2001; Bícего-Nahas
et al., 2001; Andersen and Wang, 2002; Andersen and Wang, 2003; Seebacher and Franklin,
2011). Recently it was suggested that the “aerobic scope model” (oxygen limitation
hypothesis) might not apply to *Rhinella marina* (Seebacher and Franklin, 2011), but
measurements of oxygen transport capacity and aerobic scope have not been performed at
temperatures approaching the critical/lethal limit. A critical evaluation of the “aerobic scope
model” demands that resting and maximal rate of oxygen consumption are correlated with
simultaneous cardio-respiratory measurements across a broad thermal range including
temperatures close to the upper thermal limit. The upper thermal limit of *Rhinella marina*,
assessed from loss of muscular control, is approximately 41-42°C (Stuart, 1951; Krakauer,
1970; Johnson, 1972). Heat tolerance, however, is influenced by prior acclimation
(Brattstrom, 1968).

Here we present resting and maximal oxygen consumption rates from low (10°C) to
stressfully high temperatures (40°C). To specifically address temperature sensitivity of
circulation and ventilation, we measured heart rate and blood pressure, as well as arterial
blood gases at both benign (30°C) and stressfully high temperatures (40°C). To include the
potential role of thermal acclimation, we studied animals chronically acclimated to 20, 25 and
30°C. We specifically test the hypothesis that aerobic scope decreases at high temperatures in
an air breathing ectothermic vertebrate and that this decrease is associated with reduction in
cardiovascular and ventilatory capacities. Moreover, we test the hypothesis that chronic heat
acclimation markedly improves heat tolerance through an increased oxygen transport
capacity in warm acclimated animals.

**Materials and Methods**
Experimental animals and acclimation regime

36 adult cane toads (*Rhinella marina*, formerly *Bufo marinus*) of unknown sex weighing between 60 to 170g (97 ± 4.3 g SE) were purchased from Exotic Tropicals Herpetoculture (Barbados, West Indies) and transported to University of Aarhus. To study the influence of thermal acclimation on cardio-respiratory variables as well as resting and maximal oxygen consumption rate, the toads were randomly divided into three acclimation groups (N=12 in each) shortly after arrival and kept at a 12h light cycle at constant temperatures of 20, 25 and 30°C, respectively, for a minimum of 7 weeks before the onset of experiments. During acclimation and the preceding experimental period, the animals were kept in containers with free access to fresh water and substrate for burrowing. The toads were fed mealworms and crickets twice weekly; food, however, was withheld 48h before instrumentation.

Experimental protocol

Rates of resting and maximal oxygen consumption (RMR and VO$_2$max, respectively) were measured in six toads from each acclimation group at temperatures between 10 and 40°C over a 6 month period. The order of temperatures studied was chosen at random (30, 20, 15, 10, 35, 40, 25, 36 and 38°C) to avoid possible directional acclimation effects during the acute exposures. All three acclimation groups were always measured at the same temperature in the same week and there was always at least one week between measurements at different temperatures. Before each measurement we moved toads directly from their housing temperature to the experimental temperature and let the animals settle at this temperature for approximately 24 hour before measurements of oxygen consumption rate. After measurements of oxygen consumption rate, the toads were maintained at their acclimation temperature for approximately two months before blood pressure and hematological parameters were measured at rest and during activity at 30 and 40°C, respectively. 30°C represents a “normal” high temperature (30°C), while 40°C is close to the upper lethal limit (Stuart, 1951; Krakauer, 1970; Johnson, 1972; personal observations) and may, therefore, be associated with cardiovascular collapse. Animals were taken directly from their acclimation temperature, operated, recovered at room temperature and then placed at either 30 or 40°C at random. The animals were left at the “first” experimental temperature for at least 18 hours before measurements of hematological variables and subsequently moved to
the “other” experimental temperature for 24 hours before the final set of measurements was obtained.

**Measurements of the rate of oxygen consumption in resting and active toads**

RMR and VO₂max were measured using closed respirometry using the methodology described in Withers et al., 1988 and Andersen and Wang, 2003. On the day of measurements the animals were enclosed in 3 l cylindrical chambers, placed in an incubator at constant temperature and light intensity and supplied with continuous air supply at high humidity. The toads were kept with minimal disturbance for no less than 3h to provide three consecutive measurements of RMR. To minimize the influence of spontaneous activity, the lowest RMR value was used for further analysis. Given the duration of experiments using closed respirometry these measurements represent our lowest assessment of routine oxygen consumption rate in calm and unrestrained animals (due to the limited activity and calm nature of the experimental animals we will subsequently refer to these measurements as RMR). During measurements of RMR the chambers were sealed for 60 to 160 min. Air samples were withdrawn at the beginning and end of this period and analyzed for fractional oxygen content in a gas analyzer (O₂ S-3 A; Applied Electrochemistry, Sunnyvale, CA, USA). For measurements of VO₂max, the toads were enforced to exercise for 4 min by manual rotation of the cylindrical chamber in a water bath at the experimental temperature as this procedure has been shown to maximize VO₂ in anuran amphibians (Withers et al., 1988). The fractional content of the container never fell below 18 %, and we assume that metabolism was unaffected by the altered CO₂ and O₂ concentrations.

**Arterial cannulation**

Toads were anaesthetised by immersion into a 1.0 g l⁻¹ benzocaine solution (ethyl p-amino benzoate, Sigma® E 1501) at room temperature until the corneal reflex disappeared. The femoral artery was occlusively cannulated through an incision in the hind leg, which was closed by sutures. The toads were placed under running tap water to recover for approximately 30 min; Andersen and Wang, (2002) showed that blood gases return to normal values within 6h upon a similar procedure. Each animal was subsequently transferred to an experimental container (40x30x20cm) with wet paper towels and placed in a climatic chamber, at the relevant experimental temperature for at least 18 h before blood sampling. Toads were shielded from visual and auditory disturbances during blood sampling.
**Blood pressure and heart rate**

Mean arterial blood pressure (MAP) was measured by connecting the femoral catheter to a Baxter Edward (model PX600, Irvine, CA, USA) disposable pressure transducer. The signal was amplified using an in-house built preamplifier and calibrated daily against a static water column. Signals from the blood pressure transducer were collected digitally with a BioPac MP 100 (BioPac Systems, Inc., Santa Barbara, CA, USA) at 50 Hz. Heart rate ($f_H$) was derived from the pulsatile blood pressure. A continuous recording of 3 - 8 min was used for each measurement to determine MAP and $f_H$.

**Haematological parameters and blood gases**

Arterial blood samples were taken from resting undisturbed animals, and immediately upon exhaustion after enforced activity, and analysed immediately for oxygen tension ($PaO_2$), $pH_a$, haematocrit, blood haemoglobin concentration ([Hb]a), oxygen concentration ([O2]a) and total carbon dioxide content of plasma ([CO2]pl). $PaO_2$ and $pH_a$ were measured with Radiometer (Copenhagen, Denmark) electrodes maintained at the same temperature as the animal and connected to a Radiometer PHM 73. Haematocrit was determined as the fractional red cell volume after centrifugation (12,000 rpm for 3 min) and [Hb]a was measured after conversion to cyanmethaemoglobin, applying a millimolar extinction coefficient of 10.99 at 540 nm (Zijlstra et al., 1983). Arterial $[O_2]$ was measured as described by Tucker (1967), with the correction pointed out by Bridge et al., (1979). Haemoglobin bound oxygen ($HbO_2$) was calculated as $[O_2]_a - (\alpha_{O_2} \times PaO_2)$, where $\alpha_{O_2}$ is the blood oxygen solubility determined by Christoforides and Hedley-Whyte, (1969), and haemoglobin oxygen saturation was subsequently calculated as: $HbO_2sat = HbO_2 / [Hb]$, under the assumption that all Hb was functional.

Plasma [CO2] was measured according to Cameron, (1971). Arterial PCO2 ($P_aCO_2$) and plasma [HCO3] were calculated from the Henderson-Hasselbalch equation with the plasma solubility of CO2 ($\alpha_{CO_2}$) provided by Boutilier et al., (1979), and an apparent pK derived from Heisler (1986). Plasma lactate concentrations were measured using an YSI 1500 SPORT lactate analyzer (YSI Inc. Life Sciences, Yellow Springs, Ohio, US).

**Statistical analysis**

The effects of acclimation and temperature on RMR and VO2max as well as absolute and factorial aerobic scopes were tested using a two-way Analysis of Variance (ANOVA) upon
log transformation due to unequal variance. All hematological and cardiovascular parameters were tested using a three-way ANOVA (with the factors test temperature (30 vs. 40°C); acclimation group (20, 25 and 30°C) and activity level (rest or exercise)). The level of significance was chosen at the P<0.05 level following Bonferroni correction. Values are presented as mean ± 1 S.E.M.

**Results**

*Oxygen consumption rate at rest and activity*

Resting metabolic rate at 10°C ranged between 0.19-0.22 ml kg⁻¹ min⁻¹ for the three acclimation groups and increased exponentially with temperature, reaching 1.43-2.13 ml kg⁻¹ min⁻¹ at 38°C (Table 1 and Fig. 1A). There was a significant interaction between experimental temperature and acclimation, such that RMR of toads acclimated to 20°C was significantly higher than RMR of the other acclimation groups when this was measured at 30°C. A consequence of the experimental protocol employed here is that the last measurements of oxygen consumption rate were performed on animals acclimated for more than 8 months while the first measurement were performed in animals with only 2 months acclimation. However, the order of experimental temperatures were randomized and given the limited observed effect of thermal acclimation on rate of oxygen consumption we believe that any putative effect of acclimation duration are minor.

VO₂max also increased with temperature and reached a maximum of 19.9 to 21.9 ml kg⁻¹ min⁻¹ at 38°C independent of acclimation (Table 1 and Fig. 1B). This value was not significantly larger than that measured at 36 or 40°C. Aerobic scope is presented as the absolute difference between RMR and VO₂max as well as the factorial rise between rest and activity in Figures 1C and D. Independent of acclimation, absolute aerobic scope increased from around 3 ml kg⁻¹ min⁻¹ at 10°C to almost 20 ml kg⁻¹ min⁻¹ at 38°C. Aerobic scope seemed to stabilize at the higher temperatures, and there were no significant differences in the range between 36-40°C. The pattern was considerably altered when aerobic scope is presented as the factorial increase in the rate of oxygen consumption (Fig. 1D). Factorial aerobic scope was generally higher (17-20) at temperatures from 30°C and below, while the factorial difference between maximal and resting values where around 12-15 at temperatures from 35 to 40°C. We also found a significant effect of acclimation, where the 20°C acclimated toads generally had lower factorial aerobic scope. This probably results from the higher RMR in this group.
Cardiovascular and hematological responses to activity at 30 and 40°C

Heart rate ($f_H$) at rest and during activity increased significantly with temperature (from 36.7±3.7 to 62.0±3.8 BPM; $Q_{10} = 1.7$ at rest and from 71.7±3.8 to 92.9±3.9 BPM; $Q_{10} = 1.3$ during exercise) (Fig. 2A). Mean arterial blood pressure (MAP) did not change with increased temperature, but rose significantly in response to exercise. Due to a significant interaction between experimental temperature and exercise, the effect of exercise was larger at 30°C (35.9±2.2 to 57.8±2.2 cm H$_2$O) than at 40°C (38.1±2.2 to 48.6±2.3 cm H$_2$O) (Fig. 2B). There was no effects of temperature acclimation on $f_H$ or MAP.

Haematocrit (hct) was lower in toads acclimated to 20°C than toads acclimated to 30°C, but was not affected by experimental temperature. Hct increased significantly during exercise (from 15 to 20%, Fig 3A) accompanied by a similar rise in blood oxygen concentration and [Hb] (Fig 3B). There was an indication of cellular swelling as Mean Cellular Hemoglobin Concentration (MCHC) decreased slightly with both temperature and exercise (Fig 3C).

Arterial PO$_2$ was generally above 80 mmHg in all acclimation groups and increased when temperature was increased from 30 to 40°C (Fig. 4A). Exercise was also associated with increased HbO$_2$ saturation as calculated from the ratio of the estimated amount of haemoglobin-bound oxygen and the measured Hb concentration ([HbO$_2$]/[Hb]$_{total}$). There were no significant differences in saturation in any of our three factors, such that saturation remained high irrespective of acclimation, activity level and temperature (Fig 4B).

As seen in Fig. 5A arterial pH of resting toads was similar in all acclimation groups and pH decreased significantly when temperature was increased from 30 to 40°C, due to a significant rise in arterial PCO$_2$ (12.4±0.5 to 16.7±0.7 mmHg)(Fig 4C), while plasma [HCO$_3^-$] did not change (Fig. 5C). Arterial pH fell significantly upon exercise at both temperatures reaching 7.60±0.03 and 7.51±0.03 at 30 and 40°C, respectively (Fig. 5A). This acidosis was primarily metabolic in origin as the average change in plasma [HCO$_3^-$] across all acclimation groups was a decrease from 22.3±0.73 to 13.8±0.71 mM (Fig. 5C) in response to an almost equimolar rise in plasma lactate from ~1 mM to 10 mM following exercise (Fig. 5B).
Discussion

Several recent studies on fish and aquatic invertebrates emphasize collapse of the cardio-
respiratory systems abilities to deliver adequate amounts of oxygen to the respiring tissues as
the primary determinant for upper thermal tolerance (Pörtner, 2001; Pörtner and Knust, 2007;
Pörtner and Farrell, 2008; Somero, 2010; Verberk and Calosi, 2012). While this aerobic
scope model requires much more investigation in water-breathing as well as air-breathing
vertebrates, we demonstrate here that cardio-respiratory failure is an unlikely determinant of
the upper thermal limit in the toad *Rhinella marina*. Thus, we found little evidence for
reductions in either VO$_2$max, heart rate, arterial oxygen saturation or aerobic scope at
temperatures immediately below the lethal temperature (41-42°C; Stuart, 1951; Brattstrom,
1968; Krakauer, 1970; Johnson, 1972). The “aerobic scope model” is based on the correlation
between the dwindling aerobic scope and the reduced growth and overall performance with
increased temperatures (See, Fry and Hart, 1948; Brett, 1971; McCormick et al., 1972;
Pörtner and Knust, 2007). There are several examples of clear and correlated reductions in
aerobic scope, oxygen transport capacity and fitness with elevated temperatures in fishes
(Pörtner and Knust, 2007; Nilsson et al., 2009; Eliason et al., 2011; Neuheimer et al., 2011)
and also in aquatic invertebrates (Pörtner, 2001; Somero, 2010; Somero, 2012; Verberk and
Calosi, 2012). However, there is contradicting evidence for fish studies regarding the relation
between thermal tolerance and aerobic scopa (Clark et al., 2005a; 2011; Keen and Gamperl,
2012) and aquatic invertebrates (Marshall et al., 2011) and it also seems that thermal
tolerance in air-breathing animals is determined by other factors than oxygen transport
capacity. Thus in accordance with our study on toads, the “aerobic scope model” does not
seem to apply to terrestrial insects (Klok et al., 2004; Stevens et al., 2010). These “other”
factors could include protein denaturation, effects on membrane fluidity, thermal inactivation
of enzymes at rates that exceed rates of formation and different temperature effects (Q$_10$) in
interdependent metabolic reactions (Cossins and Bowler, 1987; Schmidt-Nielsen, 1997), but
our study cannot address the relative importance of such factors.

It is possible that differences between water- and air-breathers may reside with the
larger ventilatory requirements for water breathers, which, in combination with the decreased
oxygen solubility as water temperature increases, pose larger limitations on the rate of
oxygen uptake. In addition, it has been argued that the spongy inner myocardium in fish,
being largely devoid of coronary supply, is more susceptible to decreased venous oxygen
levels at higher temperatures (Farrell and Clutterham, 2003). The anuran ventricle is also
largely spongy (Johansen and Hanson, 1968) and lacks coronary supply to the ventricle (MacKinnon and Heatwole, 1981). However, because the left side of the anuran heart receives oxygenated blood from the lungs, it is possible that myocardial oxygen delivery is unaffected by low venous oxygen levels during exercise at high temperatures.

Numerous studies have characterized the thermal dependence of cardiorespiratory functions in amphibians, including *R. marina* (See for example Whitford, 1973; Carey, 1979; Feder, 1982; Hillman et al., 1985; Hillman, 1987; Withers et al., 1988; Gamperl et al., 1999; Hedrick et al., 1999; Andersen et al., 2001; Bícego-Nahas et al., 2001; Andersen and Wang, 2002; Andersen and Wang, 2003; Andersen et al., 2003; Seebacher and Franklin, 2011 for examples). These studies (and references within) clearly show that some cardiorespiratory parameters are sensitive to both seasonal and experimental factors (Gamperl et al., 1999; Bícego-Nahas et al., 2001; Andersen et al., 2003; Seebacher and Franklin, 2011).

Nonetheless, the measurements presented here are generally in accordance with those observed in these previous studies performed at lower temperatures. However, the heart rate of active toads at 30°C measured in our study is slightly lower than those reported by Hedrick et al. (1999) and Seebacher and Franklin, (2011).

The present study is the first to report measures of metabolism and cardio-respiratory parameters in amphibians exercising above 30°C. We found high arterial O₂ saturation even at the highest temperatures indicate very low levels of R-L shunt, which is somewhat at odds with Hedrick et al., (1999), who concluded that intra-cardiac mixing increases at high heart rates. On the other hand it is also possible that the comparatively low heart rates observed in our study (see discussion above) avoided marked increases in the cardiac R-L shunt. Even though we did not measure blood flows, we can estimate a minimal required systemic stroke volume during VO₂max on basis of the measured arterial O₂ concentration and an assumption of 80% O₂ extraction. Using this approach, we estimate systemic stroke volume of 3.8, 4.1 and 2.9 ml beat⁻¹ at 40°C for 20, 25 and 30°C-acclimated toads, respectively. These estimates are slightly larger than direct measurements with Doppler flow probes (Hillman et al., 1985; Hedrick et al., 1999), and emphasizes that the arterial-venous O₂ extraction is indeed very high during exercise as reported in previous studies (Withers et al., 1988; Hedrick et al., 1999; Seebacher and Franklin, 2011). It is possible that the high oxygen extraction is facilitated by the right-shifted oxygen dissociation curve as temperature increases and pH falls due to lactic acidosis (Andersen et al., 2001).
We found no evidence for the oxygen limiting hypothesis in the ventilatory and diffusive parts of the oxygen delivery cascade. High arterial $O_2$ levels demonstrate that there is no major limitation for gas exchange across the lungs and the presumed low venous $O_2$ levels indicate that the diffusive capacity in the tissues is adequate, even during exercise at high temperatures. While exercise was associated with considerable anaerobic metabolism, the lactate levels measured in plasma were not higher at 40 compared to 30°C. However, because of the low $Q_{10}$ for heart rate between 30 and 40°C ($Q_{10} = 1.17 - 1.44$ for the three acclimation groups, respectively), it is possible that the cardiac capacity is approaching a limitation at 40°C, which may contribute to the apparent plateau of VO$_2$max and decreasing factorial aerobic scope above 36°C. Thus, V/O$_2$max did tend to decline above 36 °C, but this reduction was not statistically significant. Nonetheless, oxygen transport capacity remains more than 10-fold above resting needs at a temperature marginally below the lethal limit and this low $Q_{10}$ is therefore unlikely to be the main cause of thermal collapse. Indeed, previous studies of air-breathing vertebrates stipulate that factorial and absolute scope is retained at high temperatures (See, Whitford, 1973; Carey, 1979; Clark et al., 2005 and references within). Another prediction of the “aerobic scope” model is that lactate will start to accumulate even under routine conditions once the capacity of the oxygen transport system becomes inadequate. This was clearly not the case in our study, where blood lactate levels at rest remained at the low levels reported lower temperatures (Andersen and Wang, 2002; Andersen and Wang, 2003; Seebacher and Franklin, 2011).

Acclimation (phenotypic plasticity) has the potential to alter oxygen transport capacity and/or thermal tolerance and must be considered when evaluating putative consequences of climate change (Wang and Overgaard, 2007; Franklin and Seebacher, 2009; Hofmann and Todgham, 2010; Seebacher and Franklin, 2011). Seebacher and Franklin, (2011) recently suggested that *Rhinella marina* show compensatory acclimation that maintains aerobic scope, but aerobic scope was not measured. In our study, there were virtually no differences between acclimation groups (20, 25 or 30°C), which suggest that thermal acclimation has little impact on maximal oxygen transport capacity and aerobic scope. Also, as reported previously (Feder, 1982; Seebacher and Franklin, 2011), cold-acclimated toads had slightly elevated RMR, particularly at higher temperatures. This pattern was less obvious for VO$_2$max, where cold-acclimated toads only had elevated rate of oxygen consumption at 30°C. However, by virtue of the large factorial aerobic scope in *Rhinella marina*, small differences in resting metabolism are unlikely to have any considerable impact...
on absolute aerobic scope - which is quite similar between acclimation groups at the highest temperatures. Thermal acclimation, nevertheless, may be important for other aspects since the observed differences in RMR may reflect differences in anabolic and catabolic turnover. Curiously, Bícego-Nahas et al., (2001) reported that winter acclimation lowered resting metabolic rate in the closely related *Bufo Paracnemis*. This is the opposite pattern of the increased resting metabolic rate in the cold acclimated toads found here and in other studies (Feder, 1982; Seebacher and Franklin, 2011) and implies that seasonal effects metabolism differs from the direct influence of temperature *per se*. Nonetheless, our observation that acclimation exerted little impact on aerobic performance and aerobic scope overall does not support a mechanistic link between acclimation, oxygen transport capacity and thermal tolerance. In this respect, it is interesting that maximum critical temperature (CTmax) of anuran amphibians can change more than 4°C when acclimated to either cold (5-10°C) or warm (25-35°C) conditions (Brattstrom, 1968). For *Rhinella marina* this plasticity may be somewhat confounded with estimates of plasticity ranging from 1-5°C for different populations and acclimation treatments (Brattstrom, 1968; Krakauer, 1970; Johnson, 1972). Clearly it would be interesting to investigate the relationship between CTmax, oxygen transport capacity and acclimation in amphibian species that demonstrates a consistent large acclimation response.

In conclusion, our study clearly demonstrates that the absolute increment in VO₂ during exercise is maintained at high temperatures in a terrestrial ectothermic vertebrate. We did find a low Q₁₀ for maximal heart rate between 30 and 40°C, but lactate levels of resting animals remained low and there was ample scope to increase both heart rate and oxygen transport at the highest temperatures tolerated by this species. It is unlikely, therefore, that limited oxygen transport determines the acute upper thermal limit in toads. We propose that a mechanistic understanding of upper thermal limits in this species should be sought in the collapse of other physiological systems than those concerning oxygen transport. Although we did not demonstrate a causal relationship between oxygen transport capacity and thermal tolerance in an air-breathing vertebrate, we encourage further studies on amphibians that constitute an appropriate model to investigate the applicability of the oxygen limiting hypothesis in water and air-breathers. This could for example be studied in the same individual in the larval (water-breathing) and adult (air-breathing) stage. A number of studies in insects have, for example, indicated that the applicability of the “aerobic scope model” (oxygen limiting hypothesis) depends on the respiratory medium and respiratory mode and
that it, is therefore, more applicable to water-breathing invertebrates (Klok et al., 2004;
Stevens et al., 2010; Verberk and Calosi, 2012)

Acknowledgements. This study was funded by the Danish Research Council. Special thanks
to Heidi Meldgaard Jensen for animal husbandry as well as Rasmus Buchanan, Gitte K.
Hartvigsen and Kristian Overgaard for technical assistance.
Abbreviations

RMR Resting Metabolic Rate
[Hb4]a Arterial haemoglobin concentration
[O2]a Arterial oxygen concentration
ANOVA Analysis of Variance
CTmax Maximum critical temperature
fH Heart rate
HbO2 Haemoglobin bound oxygen
HbO2sat Haemoglobin oxygen saturation
Hct Haematocrit
MAP Mean Arterial Pressure
MCHC Mean Cellular Haematocrit Concentration
PaCO2 Arterial pCO2
PaO2 Arterial oxygen tension
pHa Arterial pH
S.E.M. Standard Error of Mean
VO2Max Maximum rate of oxygen consumption
αCO2 Plasma solubility of CO2
αO2 Plasma solubility of O2
References


Figure legends

**Figure 1.** The effect of experimental temperature on resting (A; $V\overline{O}_2$rest) and maximal (B; $V\overline{O}_2$max) oxygen consumption in *Rhinella marina* acclimated to 20, 25 or 30°C, respectively (See table 1 for values and statistics). The absolute aerobic scope (C; Aerobic scope) is calculated from the difference between $V\overline{O}_2$max and $V\overline{O}_2$rest. Factorial aerobic scope (D; $V\overline{O}_2$max/$V\overline{O}_2$rest) is calculated from the ratio between $V\overline{O}_2$max and $V\overline{O}_2$rest. Values with different letters differ significantly (P<0.05). N=5-6 for each group and error bars indicate standard error of mean (S.E.M).

**Figure 2.** Heart rate (A) and arterial blood pressure (B) measures at 30 and 40°C during rest (open) and forced activity (hatched) in *Rhinella marina*. Animals chronically acclimated to 20, 25 and 30°C are shown in blue, green and red, respectively. Asterisks indicate significant effect of activity (P<0.05) and dagger indicates overall significant effect of temperature (P<0.05). N=5-6 for each group and error bars indicate standard error of mean (S.E.M).

**Figure 3.** Haematocrit (A; Hct), hemoglobin concentration (B; [Hb]) and mean cellular hemoglobin concentration (C; MCHC) measured at 30 and 40°C during rest (open) and forced activity (hatched) in *Rhinella marina*. Animals chronically acclimated to 20, 25 and 30°C are shown in blue, green and red, respectively. Asterisks indicate significant effect of activity (P<0.05), dagger indicates overall significant effect of temperature (P<0.05) and dissimilar letters indicate effect of acclimation group. N=5-6 for each group and error bars indicate standard error of mean (S.E.M).

**Figure 4.** Arterial PO2 (A), hemoglobin saturation (B) and arterial PCO2 (C) measured at 30 and 40°C during rest (open) and forced activity (hatched) in *Rhinella marina*. Animals chronically acclimated to 20, 25 and 30°C are shown in blue, green and red, respectively. Asterisks indicate significant effect of activity (P<0.05) and dagger indicates overall significant effect of temperature (P<0.05). N=5-6 for each group and error bars indicate standard error of mean (S.E.M).
**Figure 5.** pH (A), plasma lactate (B) and bicarbonate concentration (C; [HCO₃⁻]) measured at 30 and 40°C during rest (open) and forced activity (hatched) in *Rhinella marina*. Animals chronically acclimated to 20, 25 and 30°C are shown in blue, green and red, respectively. Asterisks indicate significant effect of activity (P<0.05) and dagger indicates overall significant effect of temperature (P<0.05). N=5-6 for each group and error bars indicate standard error of mean (S.E.M).
Heart rate (BPM)

Arterial blood pressure (cm H\textsubscript{2}O)

A

Measured at 30°C

Measured at 40°C

***

†

* * *

B

Rest           Active Rest           Active

Arterial blood pressure (cm H\textsubscript{2}O)

* * *

* * *
Table 1: The effect of experimental temperature on resting and maximal oxygen consumption *Rhinella marina* acclimated to 20, 25 or 30°C. Values are average ± S.E.M of RMR (V/i2O2rest) and V/i2O2max. The effect of experimental temperature and acclimation group were tested independently for RMR and V/i2O2max using a two way ANOVA (on log transformed data). There were no significant effect of acclimation regime on V/i2O2max while RMR showed different responses between acclimation groups. Groups that differ significantly are indicated by the use of dissimilar letters.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>20°C accl</th>
<th>25°C accl</th>
<th>30°C accl</th>
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<tbody>
<tr>
<td></td>
<td>RMR</td>
<td>V/i2O2max</td>
<td>RMR</td>
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<td><strong>3.37 ± 0.25a</strong></td>
<td>0.19 ± 0.02a</td>
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<td>15</td>
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<td><strong>6.12 ± 0.68b</strong></td>
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<tr>
<td>20</td>
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<td><strong>7.77 ± 0.68b</strong></td>
<td>0.40 ± 0.05bc</td>
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<tr>
<td>25</td>
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<td><strong>12.97 ± 1.38c</strong></td>
<td>0.67 ± 0.03c</td>
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<tr>
<td>30</td>
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<td><strong>19.28 ± 1.62d</strong></td>
<td>0.76 ± 0.06cde</td>
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<tr>
<td>40</td>
<td>1.64 ± 0.16de</td>
<td><strong>17.13 ± 1.04de</strong></td>
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