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Seasonal metabolic changes in a year-round reproductively active subtropical tree-frog (*Hypsiboas prasinus*)

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ABSTRACT

Although seasonal metabolic variation in ectothermic tetrapods has been investigated primarily in the context of species showing some level of metabolic depression during winter, but several species of anurans maintain their activity patterns throughout the year in tropical and subtropical areas. The tree-frog *Hypsiboas prasinus* occurs in the subtropical Atlantic Forest and remains reproductively active during winter, at temperatures below 10 °C. We compared males calling in summer and winter, and found that males of *H. prasinus* exhibit seasonal adjustments in metabolic and morphometric variables. Individuals calling during winter were larger and showed higher resting metabolic rates than those calling during summer. Calling rates were not affected by season. Winter animals showed lower liver and heart activity level of citrate synthase (CS), partially compensated by larger liver mass. Winter individuals also showed higher activity of pyruvate kinase (PK) and lower activity of CS in trunk muscles, and higher activity of CS in leg muscles. Winter metabolic adjustments seem to be achieved by both compensatory mechanisms to the lower environmental temperature and a seasonally oriented aerobic depression of several organs. The impact of seasonal metabolic changes on calling performance and the capacity of subtropical anurans for metabolic thermal acclimatization are also discussed.

1. Introduction

Temperature affects both the rates of biochemical reactions (Hochachka, 1991) and the function of organisms at higher levels of biological organization, including physiology and behavior. Temperatures that are low relative to the mean body temperatures of ectotherms usually impair physiological and behavioral performance (Huey, 1982). The thermal dependence of organismal performance is particularly consequential in species that face significant variation in field activity temperature (Huey, 1982; Sidell and Moerland, 1989; Hochachka, 1991; Navas, 1996a) as is the case for many anuran amphibians. In these animals environmental temperatures tend to modulate body temperatures and influence among others rates of evaporative water loss, digestion, and oxygen uptake, as well as the velocity of muscle contraction, and other variables that appear to be ecologically relevant (see Rome et al., 1992; Navas et al., 2008 for reviews). Anuran locomotion and vocalization, for example, are thermally dependent and measurable behaviors that are generally believed to affect the ecological success of male anurans (Preest and Pough, 1993; Wells et al., 1996; Navas and Bevier, 2001). Finally, temperature may modulate life history, as it is known since the early studies by Moore (1939, 1940, 1949, 1952), and has been confirmed by numerous additional other studies (Berven et al., 1979; Beattie, 1987). Despite the pervasive effects of temperature on anuran behavior and physiology, a common scenario in subtropical settings is that anuran species facing significant variation in environmental temperatures through the seasons remain active all year round.

Anuran species that remain active through seasons may 1) accept passively the expected effects of temperature on physiology and behavior; 2) maintain similar levels of performance through the family of physiological adjustments collectively known as acclimatization; or 3) be active at submaximal rates so that no apparent effects of temperature can be observed on undisturbed, normally active animals. However, the ability of anuran species to acclimatize seems to vary with latitude, and previous studies have shown that, when compared with temperate counterparts, adult amphibians from tropical and subtropical regions exhibit limited or no capacity for metabolic acclimation (Feder, 1982a, 1983, 1986; Feder and Gibbs, 1982). Feder (1982b), for example, examined metabolic rates of seven tropical amphibian species, six of which did not respond to thermal acclimation, whereas one (*Rana erythraea*) exhibited a decreased metabolic rate during cold acclimation (inverse compensation). Similarly, lack of metabolic acclimation characterizes also high-altitude eurythermic tropical anurans in different families (Navas,

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1996a) and several groups of stenotherm ectotherms, both tropical and Antarctic (Tsuji, 1988; Pierce and Crawford, 1997; Weinstein and Somero, 1998; Peck and Conway, 2000; Tullis and Baillie, 2005). In contrast, more than twenty temperate amphibian species (Feder, 1982b) and other eurythermic vertebrates from temperate climates exhibit metabolic acclimation (Tsuji, 1988; Sidell and Moerland, 1989). Nevertheless, the scenario seems more complex than what this apparent dichotomy suggests, because Chang and Hou (2005) reported metabolic compensation in the adult subtropical *Rana latouchii* at particular acclimation temperatures and seasons.

Given the current state of knowledge, additional studies with subtropical anurans facing thermal variation through the year may contribute to a better understanding of the mechanisms and ecological significance of acclimatization in this taxon. Several subtropical Brazilian anurans are reproductively active all year round (Haddad and Sazima, 1992; Rossa-Ferres and Jim, 1994; Bertoluci 1998) and provide an excellent research model to investigate whether behavior varies with seasonal changes in environmental temperature or remains comparable across seasons, perhaps through acclimatization. Seasonal variation of metabolic traits is an important but generally overlooked characteristic of subtropical anurans, even though it may be widespread and extensive to other taxa of ectothermic tetrapods. Furthermore, seasonal adjustments of physiology may be related not only to temperature but also to rainfall and food supply (Chang and Hou, 2005). In this study we test the hypothesis that a year-round reproductively active subtropical tree-frog (*Hypsiboas prasinus*) maintains similar levels of behavioral performance, quantified as calling rate, despite seasonal temperature variation. If such hypothesis holds, it is likely that metabolic adjustments enhance calling performance during the colder season. Therefore, we studied the effects of season on calling rates, resting rates of oxygen consumption, and on the activity of selected enzymes from oxygen-dependent and independent pathways in different organs.

2. Materials and methods

2.1. Study animal and site

H. prasinus is a tree-frog occurring in the Atlantic Forest from south to southeastern Brazil at moderate altitudes. This species reproduces throughout the year, despite significant seasonal changes in temperature and rainfall (Haddad and Sazima, 1992; Faivovich et al., 2004; Ribeiro et al., 2005). Calling male *H. prasinus* (Anura/Hylidae) were observed and captured during summer (January 2006, $N=13$) and winter (June 2006, $N=13$) in the Serra do Japi, State of São Paulo, Brazil. Serra do Japi (46°52′W, 23°11S) is considered an ecotone of semideciduous and ombrophilous forest (Leitão-Filho, 1992), with an altitude range from 700 to 1300 m and an area of 350 km^2. In this environment summer defines a wet/warm season from October to March whereas winter is a dry/mild season from April to September (Pinto, 1992).

2.2. Behavioral observations

Calling males were located by visual inspection and observed for 30 min to quantify calling behavior. This period of observation was sufficient to evaluate the calling rate because males of this species call at relatively constant rates throughout the night. To reduce interference of observers on behavior we used red lamps and started to record behavior 10 min after an individual had been detected. All observations were performed during the period of maximum calling activity, between 1900 and 2400 h. Notes on the substrates used for calling, height above the ground, and distance from the water were recorded. The surface body temperature of some individual frogs was measured using an infrared thermometer (Instrutherm TI-900). For each individual, the total number of calls was counted and divided by sample time to estimate average calling rate during the observation period (number of calls per hour).

2.3. Animal capture and maintenance

The frogs were captured by hand and placed individually in plastic containers provided with water-soaked foam and artificial plants. Animals were then transported to the laboratory where they were kept in individual containers, exposed to natural light/dark cycles and temperatures similar to their thermal regime (20–25 °C). Animals were fasted for two days prior to the oxygen consumption measurements.

2.4. Oxygen consumption

Resting oxygen consumption rates were measured at 25 °C in fully hydrated animals using a closed respirometry system (Gomes et al., 2004). All measurements were taken during the day, between 1100 and 1500 h, when the tree-frogs were less likely to be active. The animals were placed inside well-sealed acrylic metabolic chambers (142 mL) with a piece of wet cotton. The metabolic chambers were then placed into a temperature-controlled chamber (Germinador 102G, Eletrolab), with temperature set within 25±0.2 °C. The animals were left in the chambers for 2 h to allow all individuals to assume a typical resting posture. During this period, water-saturated air was pumped through the chambers to prevent dehydration or hypoxia. Outdoor air was then pumped into the chambers for 12 min to completely wash the metabolic chambers and the chambers were closed for 2 h. Next the chambers were washed with fresh air during 12 min, and out-flowing air was drawn through a silica gel and ascarite scrubber and subsequently directed to an oxygen analyzer (Sable Systems PA-1) at a constant rate of 130 mL/min. Oxygen concentration data was captured and recorded using the Datacan data acquisition system (Sable Systems), and oxygen consumption rate was calculated by using flow-dependent equations that incorporate the integrated area under the curve of oxygen concentration *versus* time after full washing of the chamber (original method described in Bartholomew and Lighton, 1986). Animal movements were monitored by filming the metabolic chambers, and data from animals that moved during measurements were not included in the analysis (only minor posture adjustments were tolerated). Each animal was weighed to the nearest 0.01 g before and after each measurement, and the average mass was used for oxygen consumption calculations.

2.5. Tissue collection and preparation

After the oxygen consumption measurements, each animal was placed on ice and killed by decapitation followed by spinal cord pithing. The internal and external oblique muscles (collectively known as trunk muscles), heart, liver, and mixed hind limb muscles were quickly dissected, weighed (0.0001 g), transferred to cryogenic vials, and frozen in liquid nitrogen. Samples were subsequently stored at −80 °C until biochemical assays were performed. During dissections, the presence or absence of abdominal fat bodies and food remains in the gastrointestinal tract was recorded. Frozen tissues were homogenized using a Teflon-glass homogenizer (Potter-Elvehjem; Marconi Ltda) in a 1:10 dilution with homogenizing buffer at pH 7.4 containing ice-cold Imidazole 20 mM, EDTA 2 mM, NaF 20 mM, phenylmethylsulfonyl fluoride 1 mM, and 0.1% Triton X-100. The homogenates were then sonicated using a U-200S control unit (IKA-Labor Technik) in three 10-sec pulses at 50% maximum amplitude with 1-min cooling intervals, and kept ice-cold until enzymatic assays on the same day.

2.6. Enzyme assays

Pyruvate kinase (PK), lactate dehydrogenase (LDH) and citrate synthase (CS) activity level were measured as indicators of the glycolytic capacity, the capacity for lactate production, and the oxidative capacity, respectively, in the homogenates of tissue samples from heart, liver,

Table 1
Descriptive statistics of physiological, behavioral, and morphological variables of *Hypsiboas prasinus* in different seasons

Variable	Summer	Winter	Winter percentual change relative to summer
Body mass (g)	4.87±0.55	5.92±0.80	122% *
Calling rate (calls/h)	723.87±717.30 (15)	607.86±276.70	84%
Rate of oxygen consumption (mL O_2 h^{-1})	0.81±0.23	1.12±0.17	138% *
Heart mass (g)	0.012±0.002	0.018±0.004	150%
Liver mass (g)	0.086±0.017	0.139±0.027	162% *
Leg muscle mass (g)	0.67±0.09	0.85±0.13	127%
Trunk muscle mass (g)	0.38±0.09	0.52±0.09	137%
Activity of PK trunk (U/g wet mass)	71.19±26.40	106.29±25.12	149% *
Activity of PK leg (U/g wet mass)	106.68±37.62	102.32±32.06	96%
Activity of PK liver (U/g wet mass)	27.51±4.29	5.51±3.28	20% *
Activity of PK heart (U/g wet mass)	69.32±23.31	79.06±9.40	114%
Activity of LDH trunk (U/g wet mass)	142.02±42.58	148.12±20.41	104%
Activity of LDH leg (U/g wet mass)	327.33±105.24	227.98±61.06	70% *
Activity of LDH liver (U/g wet mass)	53.08±9.93	57.36±13.47	108%
Activity of LDH heart (U/g wet mass)	256.03±65.00	220.90±24.18	86% *
Activity of CS trunk (U/g wet mass)	50.73±18.63	41.60±16.61	82%
Activity of CS leg (U/g wet mass)	6.75±2.50	9.00±1.77	133%
Activity of CS liver (U/g wet mass)	23.44±5.07	5.92±1.12	25% *
Activity of CS heart (U/g wet mass)	94.13±26.36	45.58±5.11	48% *

Values are given as mean±standard deviations for $N=13$ animals, except where shown in parenthesis. * Significant differences between seasons according to ANOVA from Table 4. PK—pyruvate kinase, LDH—lactate dehydrogenase, CS—citrate synthase.

trunk muscles, and hindlimb muscles. Maximum activity of these enzymes were measured at 25 °C by following the changes in NADH absorbance at 340 nm (for PK and LDH), or that in DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) at 412 nm (for CS), under substrate saturation and no inhibitory conditions, using a spectrophotometer DU-70 (Beckman) equipped with a temperature controller (Peltier, Beckman). All assays were performed in duplicate and the results were expressed in micromoles of substrate converted to product per minute (equivalent to the unit U) per gram of tissue mass (U/g wet mass).

Enzyme protocols followed those described in Bergmeyer (1983), with minor modifications: *PK* (E.C. 2.7.1.40)—Imidazole–HCl 100 mM as final concentration (pH 7.0), $MgCl_2$ 10 mM, KCl 100 mM, ADP 2.5 mM, frutose1,6-bisphosphate 0.02 mM, NADH 0.15 mM, LDH 12.1 U mL^{-1}, tissue sample homogenate in a 1:10 extra dilution for heart, trunk and leg muscles samples, or in the raw homogenates for liver samples, and phospho(enol)pyruvate 2.5 mM (omitted from control); *LDH* (E.C. 1.1.1.27)—Imidazole–HCl 100 mM (pH 7.0); dithiothreitol 5 mM; NADH 0.15 mM, tissue sample homogenate in a 1:20 extra dilution for liver, trunk and leg muscles samples, or in a 1:30 extra dilution, for heart samples, and pyruvate 1 mM (omitted from control); *CS* (E.C. 4.1.3.7)—Tris–HCl 50 mM (pH 8.0); DTNB 0.1 mM; acetyl-CoA 0.2 mM and tissue sample homogenate in a 1:10 extra dilution for liver and heart samples, or in a 1:30 extra dilution, for trunk and leg muscles samples, and oxaloacetate 0.9 mM (omitted from control).

Each assay was run for 3 min before (control) and after adding the substrate, and the changes in absorbance were recorded. All assays were conducted in a final volume of 700 μL, and the enzyme activities were calculated based on the absorbance coefficient for NADH (6.22 10^2 L mol^{-1} mm^{-1}) or DTNB (13.6 10^2 L mol^{-1} mm^{-1}) and the distance covered by the light beam on the assay (10 mm for all assays).

2.7. Data analysis

The simultaneous effects of body mass and season on calling rate, VO_2, organ masses, and enzymatic activities were assessed by ANCOVA on $\log_{10}$-transformed data, using season as an independent variable and body mass as a covariate. Standard least-square linear regressions were used to test how different behavioral, morphometric, and metabolic variables scaled with body mass. All variables were $\log_{10}$-transformed prior to allometric regression analyses. When a significant relationship existed between dependent variables and body mass, residuals were calculated and used in subsequent analyses. The relationship between all behavioral, morphometric, and metabolic variables was determined by Pearson product moment correlation coefficients. All statistical analyses were performed via Statistica (Statsoft, Tulsa, OK) and SPSS 5.0 (SPSS, 1992).

3. Results

3.1. Field measurements, calling behavior, and dissection observations

In the summer, males were found calling from the herbaceous vegetation around a large permanent lake on perches from 0.1 to 0.8 m above substrate level, and at 0.3 to 1.5 m from the water body. During the winter, calling males occupied the same microhabitat (perches 0.1 to 1.0 m above substrate level located between 0.1 and 0.6 m from the water body). Summer body surface temperatures were 18.9±0.63 °C ($N=13$, mean±SD) whereas winter counterparts averaged 11.4± 1.00 °C ($N=13$, mean±SD). Mean calling rates were somewhat higher in summer frogs (Table 1) but this trend was not statistically significant (Table 4). Overall, the social environment seemed comparable across season, and the choruses were composed by about 30 calling males per night (females were not noticed during the behavioral observations). Both summer and winter frogs had food remains in the gastrointestinal tract, but only winter frogs exhibited well-defined fat bodies located near the last portion of the proximal intestine.

3.2. Allometry

Both organ mass ($0.74<\beta<0.87$) and resting metabolic rate ($M_b^{0.49}$) displayed a positive allometric relationship (Table 2). The activity of some enzymes also scaled allometrically: the activity of PK was positively related to the body mass in trunk muscles ($M_b^{0.40}$) and negatively related to the body mass in liver ($M_b^{-0.62}$). The activity of LDH related

Table 2
Regression equations for the effect of body mass on different variables

Variable	A±SEM	B±SEM	R^2	P
Calling rate	2.743±1.427	−0.020±0.204	0.000	0.914
Rate of oxygen consumption ($\dot{V}_{O2}$)	−0.705±0.244	0.491±0.178	0.241	**0.0109**
Heart mass	−2.876±1.470	0.821±0.116	0.675	**<0.0001**
Liver mass	−2.059±0.203	0.741±0.137	0.549	**<0.0001**
Leg muscle mass	−0.862±0.083	0.877±0.098	0.770	**<0.0001**
Trunk muscle mass	−1.240±0.154	0.761±0.132	0.579	**<0.0001**
Activity of PK trunk	1.166±0.354	0.399±0.187	0.159	**0.044**
Activity of PK leg	2.478±0.376	−0.260±0.197	0.066	0.204
Activity of PK liver	3.973±0.758	−0.620±0.160	0.384	**0.0007**
Activity of PK heart	1.575±0.272	0.206±0.200	0.043	0.312
Activity of LDH trunk	2.034±0.222	0.107±0.203	0.012	0.602
Activity of LDH leg	2.534±0.342	−0.070±0.204	0.005	0.735
Activity of LDH liver	1.330±0.183	0.411±0.186	0.169	**0.037**
Activity of LDH heart	2.301±0.209	0.065±0.204	0.004	0.753
Activity of CS trunk	1.659±0.406	−0.014±0.204	0.000	0.944
Activity of CS leg	0.191±0.549	0.240±0.198	0.058	0.237
Activity of CS liver	2.973±0.587	−0.555±0.170	0.308	**0.003**
Activity of CS heart	2.956±0.310	−0.604±0.163	0.365	**0.011**

Significant P values (<0.05) are in bold.
PK—pyruvate kinase, LDH—lactate dehydrogenase, CS—citrate synthase.

Table 3
Correlations among physiological, behavioral, and morphological variables in *Hypsiboas prasinus*

Variable	Calling rate	PK leg	PK heart	LDH trunk	LDH leg	LDH heart	CS trunk	CS leg	Heart mass	Liver mass	Leg mass	Trunk mass	PK trunk	PK liver	LDH liver	CS liver	CS heart	$\dot{V}_{O2}$
Body mass	−0.15	−0.25	0.19	0.04	−0.06	0.07	0.02	0.15	0	−0.04	0.01	−0.03	−0.01	0.01	−0.00	0.02	0.02	0.00
Calling rate		0.13	0.15	0.14	0.22	0.02	−0.21	−0.08	−0.04	−0.05	−0.04	−0.24	−0.01	0.02	−0.25	0.02	0.24	0.00
PK Leg			**−0.42**	−0.19	0.10	**−0.39**	−0.21	0.02	−0.18	0.15	−0.05	−0.05	0.03	−0.14	−0.11	−0.13	0.01	−0.15
PK heart				**0.54**	−0.31	**0.45**	0.18	0.08	**0.39**	−0.00	0.04	0.34	−0.04	−0.18	−0.11	−0.08	− 0.07	0.04
LDH trunk					0.21	0.38	−0.08	0.35	0.09	−0.03	−0.22	−0.11	0.16	0.00	0.37	0.06	0.16	−0.28
LDH leg						0.06	0.08	0.03	−0.25	−0.15	−0.27	−0.38	−0.13	**0.46**	**0.35**	**0.54**	**0.63**	**−0.43**
LDH heart							0.17	0.00	−0.00	−0.35	−0.26	−0.07	0.04	0.34	0.13	**0.47**	**0.52**	−0.30
CS trunk								−0.13	0.17	−0.21	0.22	0.23	−0.13	0.14	−0.08	0.35	0.15	−0.23
CS Leg									0.27	**0.62**	−0.25	0.19	**0.57**	**−0.46**	0.29	−0.39	−0.31	0.29
Heart mass										**0.43**	0.12	**0.61**	−0.06	−0.30	−0.07	−0.35	**−0.41**	0.18
Liver mass											−0.17	0.35	0.35	**−0.54**	0.16	**−0.72***	**−0.59**	0.36
Leg mass												0.24	−0.09	−0.02	**−0.64***	−0.21	−0.25	0.13
Trunk mass													0.08	−0.27	−0.38	−0.35	−0.31	**0.39**
PK trunk														**−0.51**	0.27	**−0.41**	−0.17	0.23
PK liver															−0.06	**0.78***	**0.69***	−0.33
LDH liver																0.14	0.05	−0.22
CS liver																	**0.83***	**−0.41**
CS heart																		**−0.48**

$\dot{V}_{O2}$, heart mass, liver mass, leg muscle mass, trunk muscle mass, activity of PK in trunk muscle and liver, activity of LDH in liver, and activity of CS in liver and heart are residuals of log–log body mass regressions. Calling rate, body mass, activity of PK in leg muscle and heart, activity of LDH in trunk muscle, leg muscle, and heart, and activity of CS in trunk and leg muscles are absolute values. Activity of LDH in heart and leg muscle is expressed as $\log_{10}$ of the absolute values.
Significant unadjusted correlations are indicated in boldface; values of $r>0.39$ are significant to 0.05; absolute $r>0.53$ are significant to 0.01. Significant correlations after a sequential Bonferroni correction for multiple simultaneous tests within sub-tables (Rice, 1989), are indicated with asterisks; absolute values of $r>0.64$ remain significant at the 'tablewide' P of 0.05. Abbreviations as in Table 1.

allometrically to liver mass ($M_b^{0.41}$), whereas the activity of CS scaled negatively with liver mass ($M_b^{-0.55}$) and heart mass ($M_b^{-0.60}$; Table 2).

3.3. Effects of body mass and seasonality on physiological variables

A number of seasonal changes were observed, as reported in Tables 1 and 4. Compared to summer frogs, winter animals were heavier (body mass 18% greater, $T_{2,24}=-3.88$, $P=0.0007$), had larger livers and exhibited higher resting metabolic rates. A number of metabolic enzymes exhibited reduced activity in winter frogs, including liver PK, leg muscle and hearth LDH, and heart and liver CS. In contrast, trunk muscle PK was higher in winter frogs. Body mass and season did not exhibit any significant interaction involving physiological variables.

3.4. Correlation among variables

Some metabolic and morphometric variables covaried after removing the effects of body mass (Table 3). For example, larger livers exhibited lower activity of CS, the activity of CS in liver and heart were tightly correlated, and the activity of liver PK correlated to the activity of liver and heart CS. Resting metabolic rates also covaried with morphometric and enzymatic variables: $\dot{V}_{O2}$ covaried positively with the mass of trunk muscles and negatively with the activity of CS in liver and heart.

4. Discussion

4.1. Seasonal variation in calling performance: social modulation or physiological limitation?

Calling performance is expected to decrease with decreasing temperatures (Prestwich, 1994), particularly in species that sustain calling behavior near their maximal performance (Navas, 1996b). Consequently, to sustain near-maximal calling rates during the winter, anurans would require the maintenance of ATP cycling capacity of trunk muscles. Accordingly, Rogers et al. (2007) found greater activity level of metabolic aerobic enzymes (COX, cytochrome c oxidase, and β-hydroxyacyl CoA dehydrogenase) in trunk muscles from cold-acclimated male brown-striped swamp frog (*Limnodynastes peronii*), from Australia. Males in species characterized by low calling rates might be less sensitive to temperature (Navas, 1996b) and probably do not require acclimatory adjustments in trunk muscle aerobic capacity. The calling rates of *H. prasinus* are extremely variable independently of season, and overlap between males calling during summer and winter (Fig. 1). Therefore, the calling rates of many individuals may be submaximal, a hypothesis that is supported by the lower CS activity in heart and trunk muscles during the winter.

Most male *H. prasinus* engaged in counter-calling during observations, occupying calling sites not more than 0.5 m apart from each other. Interestingly, the three males sustaining higher calling rates in Fig. 1 remained more distant from neighbors (around 2 and 3 m apart). Gerhardt et al. (2000) suggested that call matching between interacting males probably allows males to make themselves as attractive to females as those of their nearest rivals, without expending energy unnecessarily. If this is the case, counter-calling individuals might not need to attain the level of calling effort necessary for temperature effects to manifest (Wong et al., 2004). Consequently, variation in calling rates among individuals of *H. prasinus* seems modulated by social interactions rather than limited by physiological capacity.

4.2. Metabolic adjustments to sustain calling activity during winter

Although male *H. prasinus* showed slightly lower trunk muscle CS activity during winter, the concomitant 33% higher activity of PK could

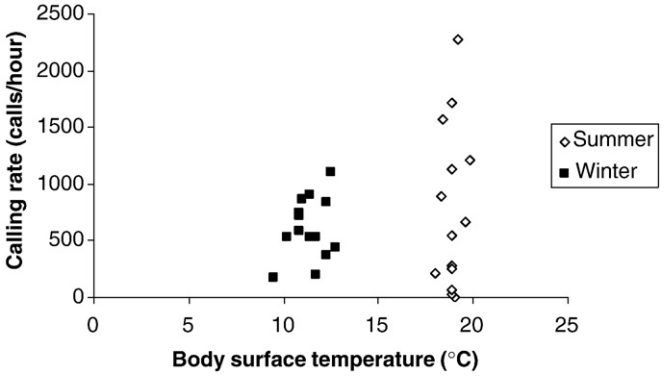

Fig. 1. Calling rates of male *Hypsiboas prasinus* in summer and winter.

enhance the carbohydrate utilization in this tissue (James et al., 2005). Such increased glycolytic flux would provide more pyruvate as a substrate for acetyl-CoA formation, as well as intermediate compounds of the Krebs cycle (see Hochachka and Somero, 2002). Glycolysis is regarded as less thermally dependent than the more complex oxidative pathways such as β-lipid oxidation (Bennett, 1994), so that the use of carbohydrates may be advantageous during winter, when temperature naturally reduces reaction rates. Additionally, anaerobic pathways in trunk muscles may contribute significantly to maintain energetic homeostasis in anurans that call sporadically and at low rates (Carvalho, 2004; Carvalho et al., 2008). High activity of trunk muscle PK, together with conserved LDH activity, would enhance glycolysis through the anaerobic pathway during winter. Interestingly, the same pattern of preponderance of glycolysis was observed in pedipalpal and heart muscles from a tropical scorpion (*Heterometrus fulvipes*) after cold acclimation (Kalarani et al., 1991). The lower total aerobic capacity of liver during the winter corroborates studies showing that short bouts of calling are mainly supported by energetic substrates accumulated in the skeletal fibers, rather than in extra-muscular stores such as the liver (Fournier and Guderley, 1993; Bevier, 1997; Carvalho et al., 2008).

4.3. Seasonal adjustments in resting oxygen consumption rates

The higher resting metabolic rates at the experimental temperature of *H. prasinus* during winter could be interpreted as a metabolic compensation to the lower body temperatures of the animals during this season (Spicer and Gaston, 1999). The hypothesis of a compensatory mechanism is favored by the fact that animals are larger during the winter, and the allometric relationship for resting metabolic rate is lower than 1.0 ($M_b^{0.49}$). Similar patterns of cold metabolic compensation in anurans have been described across seasons at the intrapopulational (Chang and Hou, 2005), and intrageneric (Gomes, 2002; Gomes et al., 2004) variation. Metabolic compensation has been reported also across species from different latitudes (Walton, 1993; Gomes, 2002), and for tropical species from different altitudes (Navas, 1996a). The cold metabolic compensation in ectotherms has been loosely interpreted as adaptive, and perhaps allows for more immediate onset of activity at low temperatures (Packard, 1972; Fitzpatrick and Atebara, 1974; Rome et al., 1992). An alternative point of view, however, is that higher resting metabolic rates at lower temperatures would add on maintenance costs without any clear benefit for fitness, and should be negatively selected in nature (Rogers et al., 2007). According to these authors, a cold metabolic compensation would be expected only for the aerobic capacity of some organs, and in situations clearly associated with increased fitness, such as calling muscles in vocally active males. Finally, another alternative explanation for higher maintenance costs during winter is that it results from a genetic and functional coupling between aerobic capacity and maintenance costs (Bennett and Ruben, 1979), along with a compensatory cold acclimation of aerobic capacity during winter. The extent of such coupling, however, remains controversial in the literature (Rezende et al., 2004; Gomes et al., 2004; Sadowska et al., 2005).

4.4. Mechanisms of seasonal adjustments in resting oxygen consumption rates

Our study allows for some insights regarding the mechanisms responsible for higher winter metabolic rates in *H. prasinus*. Although not statistically significant, the 25% higher activity of CS in leg muscles during the winter, without a significant change in leg muscle mass (13.83% and 14.29% of body mass during summer and winter, respectively) could contribute to the winter higher VO_2, assuming that a higher tissue aerobic capacity implies a higher cost of maintenance (Bennett and Ruben, 1979). Skeletal muscles contribute to a significant portion of the body mass of anurans. Therefore, and despite low specific oxygen consumption rates during resting, this tissue may enhance winter resting metabolic rates (Zurlo et al., 1990; Rolfe and Brown, 1997; Rogers et al., 2004). The higher activity of CS in leg muscles found in winter *H. prasinus* is consistent with the increased mitochondrial content in *Triceps brachii* after low temperature acclimation in frogs reported by Ballantyne and George (1978). Both findings suggest higher aerobic capacity in locomotory muscles, a possibly widespread compensatory mechanism for activity at low temperatures in anurans. The seasonal maintenance of the aerobic capacity of trunk muscles in *H. prasinus* is also consistent with previous studies showing that changes in the aerobic capacity of this tissue correlate better with calling rates than with activity temperature (Ressel, 2001).

Tissues usually characterized by high contributions to resting metabolic rates, such as the liver and the heart (Rolfe and Brown, 1997), seem metabolically depressed in winter *H. prasinus*. However, the remarkable depression of the liver aerobic capacity during the winter, illustrated by the reduction in CS activity, seems compensated by an increase in organ mass. This increase in liver mass is likely to be compensatory and circumvent allometric effects in the opposite direction ($M_b^{0.74}$). Higher resting metabolic rates accompanied by higher specific liver masses have also been described for species of tree-frogs from the genus *Scinax* active at lower temperatures, when compared to species active at higher temperatures (Gomes, 2002; Gomes et al., 2004), corroborating the argument of a functional relationship between these variables. Note also that the activities of CS

Table 4
ANOVA for calling rate, VO_2, organ masses, and enzymatic activities of *Hypsiboas prasinus* using season as independent variable and body mass as covariate

Variable	Intercept	Body mass	Season	Corrected model	R^2
Calling rate	$F_{(1,23)}=5.941$ $P=0.023$	$F_{(1,23)}=1.015$ $P=0.324$	$F_{(1,23)}=2.092$ $P=0.162$	$F_{(2,23)}=1.052$ $P=0.365$	0.084
$\dot{V}_{O2}$	$F_{(1,23)}=0.852$ $P=0.366$	$F_{(1,23)}=0.655$ $P=0.427$	$\mathbf{F_{(1,23)}=5.674}$ $\mathbf{P=0.026}$	$\mathbf{F_{(2,23)}=7.391}$ $\mathbf{P=0.003}$	0.391
Log heart	$\mathbf{F_{(1,23)}=210.929}$ $\mathbf{P<0.0001}$	$\mathbf{F_{(1,23)}=21.256}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=2.407$ $P=0.134$	$\mathbf{F_{(2,23)}=27.575}$ $\mathbf{P<0.0001}$	0.706
Log liver	$\mathbf{F_{(1,23)}=54.171}$ $\mathbf{P<0.0001}$	$\mathbf{F_{(1,23)}=7.777}$ $\mathbf{P=0.010}$	$\mathbf{F_{(1,23)}=13.911}$ $\mathbf{P=0.001}$	$\mathbf{F_{(2,23)}=29.395}$ $\mathbf{P<0.0001}$	0.719
Log leg muscles	$\mathbf{F_{(1,23)}=54.689}$ $\mathbf{P<0.0001}$	$\mathbf{F_{(1,23)}=38.728}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=1.270$ $P=0.271$	$\mathbf{F_{(2,23)}=41.169}$ $\mathbf{P<0.0001}$	0.782
Log trunk muscles	$\mathbf{F_{(1,23)}=29.278}$ $\mathbf{P<0.0001}$	$\mathbf{F_{(1,23)}=12.731}$ $\mathbf{P=0.002}$	$F_{(1,23)}=2.419$ $P=0.133$	$\mathbf{F_{(2,23)}=18.695}$ $\mathbf{P<0.0001}$	0.619
PK trunk muscles	$\mathbf{F_{(1,23)}=18.265}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=0.083$ $P=0.775$	$\mathbf{F_{(1,23)}=5.575}$ $\mathbf{P=0.027}$	$\mathbf{F_{(2,23)}=5.491}$ $\mathbf{P=0.011}$	0.323
PK leg muscle	$\mathbf{F_{(1,23)}=31.939}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=2.502$ $P=0.127$	$F_{(1,23)}=0.826$ $P=0.373$	$F_{(2,23)}=1.259$ $P=0.303$	0.099
PK liver	$\mathbf{F_{(1,23)}=6.601}$ $\mathbf{P=0.017}$	$F_{(1,23)}=0.473$ $P=0.499$	$\mathbf{F_{(1,23)}=50.959}$ $\mathbf{P<0.0001}$	$\mathbf{F_{(2,23)}=48.531}$ $\mathbf{P<0.0001}$	0.808
PK heart	$\mathbf{F_{(1,23)}=28.694}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=0.000$ $P=0.989$	$F_{(1,23)}=1.664$ $P=0.210$	$F_{(2,23)}=1.382$ $P=0.271$	0.107
LDH trunk muscles	$\mathbf{F_{(1,23)}=54.030}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=0.000$ $P=0.983$	$F_{(1,23)}=0.359$ $P=0.555$	$F_{(2,23)}=0.316$ $P=0.732$	0.027
LDH leg muscles	$\mathbf{F_{(1,23)}=22.319}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=3.828$ $P=0.063$	$\mathbf{F_{(1,23)}=12.804}$ $\mathbf{P=0.002}$	$\mathbf{F_{(2,23)}=6.490}$ $\mathbf{P=0.006}$	0.361
LDH liver	$\mathbf{F_{(1,23)}=26.327}$ $\mathbf{P<0.0001}$	$\mathbf{F_{(1,23)}=4.365}$ $\mathbf{P=0.048}$	$F_{(1,23)}=0.408$ $P=0.530$	$F_{(2,23)}=2.586$ $P=0.097$	0.184
LDH heart	$\mathbf{F_{(1,23)}=59.900}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=2.813$ $P=0.107$	$\mathbf{F_{(1,23)}=4.911}$ $\mathbf{P=0.037}$	$F_{(2,23)}=2.515$ $P=0.103$	0.179
CS trunk muscles	$\mathbf{F_{(1,23)}=4.589}$ $\mathbf{P=0.043}$	$F_{(1,23)}=1.236$ $P=0.278$	$F_{(1,23)}=3.372$ $P=0.079$	$F_{(2,23)}=1.689$ $P=0.207$	0.128
CS leg muscles	$F_{(1,23)}=1.441$ $P=0.242$	$F_{(1,23)}=0.001$ $P=0.977$	$F_{(1,23)}=2.123$ $P=0.159$	$F_{(2,23)}=1.832$ $P=0.183$	0.137
CS liver	$\mathbf{F_{(1,23)}=8.367}$ $\mathbf{P=0.008}$	$F_{(1,23)}=1.617$ $P=0.216$	$\mathbf{F_{(1,23)}=189.613}$ $\mathbf{P<0.0001}$	$\mathbf{F_{(2,23)}=142.121}$ $\mathbf{P<0.0001}$	0.925
CS heart	$\mathbf{F_{(1,23)}=67.061}$ $\mathbf{P<0.0001}$	$F_{(1,23)}=0.273$ $P=0.606$	$\mathbf{F_{(1,23)}=46.711}$ $\mathbf{P<0.0001}$	$\mathbf{F_{(2,23)}=43.359}$ $\mathbf{P<0.0001}$	0.790

Significant P values (<0.05) are in bold.
PK—pyruvate kinase, LDH—lactate dehydrogenase, CS—citrate synthase.

in liver and heart are positively correlated and both organ masses are negatively correlated with their activity of CS (Table 3). These results suggest that mitochondrial density of both organs are lower during the winter (heart CS activity during winter drops to half the summer values), and partially compensated by larger organ masses (although it is not statistically significant for the heart—Table 4).

The possible functional consequences of lower liver aerobic capacity in winter in *H. prasinus* remain conjectural. One possibility is that the seasonal decrease in these values reflects an ancient phylogenetic pattern associated with metabolic depression during the cold season, even though this species remains fully active during the winter. Another possibility (not necessarily exclusive) is that the lower liver specific aerobic capacity is associated with a concomitant large increase in liver lipid and glycogen, so that the lower aerobic capacity of liver is an artifact of enzyme dilution, or an active inhibition of catabolic pathways promoting the store of energetic reserves. If so, the liver would work as a large buffer energetic reserve for replenishing energy for other tissues, such as trunk muscles during the winter, in a more long-term period (Carvalho et al., 2008). Another large extra-muscular energetic reserve found only during the winter was the fat bodies located around the proximal intestine. Such energetic reserves could be accumulated during the autumn, and resemble other species of ectothermic tetrapods that actually remain dormant during the winter (Souza et al., 2004). Analyses of energetic reserves in frozen tissues from these same individuals will be considered in the next steps of the present study. Other organs have been described in the literature as important contributors to the resting oxygen consumption rates, such as brain, kidneys, gastro-intestinal and reproductive tracts (Rolfe and Brown, 1997), and they can be considered for future analysis in the context of the higher winter resting metabolic rates in *H. prasinus*.

4.5. Relevance of our results to the discussion of acclimatory capacity of subtropical ectotherms

One limitation of our study is that we were unable to compare thermal performance curves for both seasons. Such approach would be ideal (Angilletta, 2006), yet impractical given the ambitious objectives of the study and the limited amount of time that animals could be maintained in captivity. As a consequence, we could not evaluate the actual effects of the seasonal metabolic adjustments observed in *H. prasinus*. Despite this limitation, our results show clearly that a number of very relevant metabolic shifts occur between seasons, and that natural behavior remains constant. These two findings are consistent with the hypothesis of acclimatization. Note also that *H. prasinus* is phylogenetically related to other species of Neotropical tree-frogs that sustain calling activity during winter in Southern Brazil or that occupy environments characterized by more drastic temperature conditions, such as the Andes (Faivovich et al., 2004). Therefore, comparative studies seem promising to understand the phylogenetic patterns of diversification of these metabolic variables along the evolutionary history of this group of tree-frogs. Our findings corroborate statements from Chang and Hou (2005), who claimed that the actual state of restricted data collection to short acclimatory periods on subtropical amphibians and reptiles without regard to possible patterns of seasonal variation does not permit generalizations about their ability for metabolic thermal acclimation. Moreover, comparative studies dedicated to understanding the diversity of metabolic seasonal variation and acclimation ability in these groups must be carefully designed to incorporate information on their phylogenetic history and biogeography.

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