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The effect of short- and long-term fasting on digestive and metabolic flexibility in the Andean toad, *Bufo spinulosus*

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SUMMARY

Hibernation in ectothermic animals was historically considered as a simple cold-induced torpor state resulting from the inability to maintain a high body temperature at low ambient temperatures. During the last decades this vision changed and nowadays there is a myriad of studies showing that hibernation implies different adjustments at the genetic, molecular, biochemical and cellular levels. However, studies oriented to evaluate changes of whole organism structure and physiology still are scarce, which is particularly true for amphibians that hibernate on land. Accordingly, in the Andean toad (*Bufo spinulosus*), we investigated the effect of short-term fasting and hibernation on the hydrolytic activity of digestive enzymes, histology of the small intestine, gross morphology of digestive and other internal organs and standard metabolic rate. Based on the pattern of size variation, internal organs may be grouped into those that were affected by both season and feeding condition (small intestine, stomach and liver), those that were only affected by season (fat bodies), those that were only affected by feeding condition (kidneys) and, finally, those that did not change between the three groups (large intestine, heart and lungs). Hydrolytic activity of maltase, trehalase and aminopeptidase-N followed the same pattern of variation (feeding>fasting>hibernating toads), although the change for the latter enzyme was less noticeable than for the disaccharidases. Enzymatic adjustments were correlated with changes in small intestine histology: villus and enterocyte height increased from hibernating to fasting and more markedly from fasting to feeding toads. Metabolic rate decreased during hibernation to 7.8% (at 5°C) and 13.6% (at 15°C) of summer values, which is one of the highest metabolic depressions reported for any ectothermic vertebrate. Our results suggest that amphibian persistence in highly seasonal environments is related to a large capacity of phenotypic flexibility at different organisational levels; an ability that may be related to the extensive ranges of temporal existence and geographic distribution of these vertebrates.

Key words: Andean toad, *Bufo spinulosus*, digestive physiology, energetics, enzymes, fasting, histology, hibernation, metabolic rate, organ size, phenotypic flexibility.

INTRODUCTION

Phenotypic flexibility refers to reversible changes in the traits of organisms due to changes in internal or external environmental conditions (Piersma and Drent, 2003). These adjustments may imply different levels of biological organisation (i.e. biochemical, structural, physiological, behavioural and/or life-history traits) and are usually considered as adaptations to environmental variability. In a nutshell, empirical evidence on the adaptive value of phenotypic flexibility belongs to: (1) a congruent correlation between phenotypic changes and environmental variation, which results in an increase in the fitness of the organisms (Agrawal, 1998; Relyea, 2003; Stomp et al., 2008); (2) the identification of environmental cues used by organisms to anticipate environmental changes (Denver et al., 1998; Smith, 2000); (3) the observation, in some experimental species models, that fixed (mutant) phenotypes have lower fitness than plastic (wild) phenotypes, when evaluated through a set of environments (Callahan et al., 1999; Pigliucci and Schmitt, 1999); (4) the existence of additive genetic variation for flexibility (Scheiner, 1993; Schlichting, 1986), and the fact that flexibility is hereditable (Brommer et al., 2007; Nussey et al., 2005) and able to respond to natural and artificial selection regimens (Garland and Kelly, 2006; Scheiner, 2002); and (5) a positive correlation between the amount of flexibility displayed by different species and the magnitude of environmental variability in the habitats where they evolve (e.g. Addo-Bediako et al., 2000; Naya et al., 2008a). Consequently, current knowledge indicates that phenotypic flexibility is a widespread phenomenon in nature that may help organisms to cope with the impacts of environmental variations.

One of the most pervasive changes in environmental conditions is that associated with the change of the seasons during the year. At least in temperate terrestrial habitats, winter poses a variety of selective pressures, the most important being the reduction in food availability and quality as well as the presence of extremely low temperatures (Wunder, 1984). Accordingly, animals show a number of responses to winter pressures, which may be grouped into two major strategies: (1) avoidance, which implies mechanisms for reducing the magnitude of winter conditions such as migration, microclimate selection and freeze avoidance; and (2) resistance, which implies mechanisms for combating winter pressures, including an increase in metabolic rates and thermogenesis (resting and maximum metabolism as well as non-shivering thermogenesis) and increased activity.

Among ectothermic vertebrates inhabiting seasonal and winter snowy environments, overwintering is usually related to hibernation, i.e. with a state of reduced metabolism and activity that allows animals to use their energy reserves efficiently (Pinder et al., 1992).

Historically, hibernation in these animals was visualised as a simple cold-induced torpor resulting from an inability to maintain a high body temperature at low ambient temperatures (Grenot et al., 1996). However, during the last two decades it has been recognised that hibernation in ectotherms is related to several phenotypic changes, comparable but not identical with those observed among hibernating endotherms. Hibernation in ectotherms may imply some of the following adjustments: (1) regulation in the expression of genes related to control processes (e.g. cell cycle arrest), production of molecules to preserve cellular metabolism (e.g. antioxidant enzymes, protease inhibitors, chaperone proteins) and production of molecules for species- or tissue-specific functions (e.g. urea cycle enzymes, antifreeze proteins, cryoprotectant); (2) reversible post-transcriptional modification of metabolic enzymes and functional proteins that allows, among other things, a reorganisation of metabolic priorities; (3) changes in organelles and cell membrane structure, which allow adjustments in volume and electrochemical gradients through them; (4) suppression of cell proliferation, decreasing the use of energy for growth and development; and (5) redistribution of blood flow to different tissues and adjustments in whole organism oxygen conductance and water balance (Storey and Storey, 2004; Storey and Storey, 2007; Tattersall and Ultsch, 2008). Beyond this increased interest in hibernation of ectothermic species, there is still a scarcity of studies evaluating other levels of biological organisation, such as whole organism structure (e.g. changes in the size of organs) and physiology (e.g. changes in metabolic rate). This is particularly true for the case of amphibians that hibernate on land, for which very few studies have been focused on these levels, contrasting with the situation for amphibians that hibernate submerged under water (Boutilier et al., 1997; Boutilier et al., 1999; Donohoe et al., 1998; Tatersall and Boutilier, 1997; Ultsch et al., 2004) and amphibians that aestivate (Abe, 1995; Cramp and Franklin, 2003; Cramp and Franklin, 2005; Cramp et al., 2005; Fuery et al., 1998; Secor, 2005; Withers and Thompson, 2000).

In the present study, we examined phenotypic flexibility in the energy acquisition and expenditure process in the hibernating Andean species *Bufo spinulosus* Wiegmann 1834. Specifically, we examined the effect of short-term fasting and hibernation on the hydrolytic activities of digestive enzymes, the histology of the small intestine, the gross morphology of digestive and other internal organs and, finally, the whole organism metabolic rate. The main questions that we aimed to answer were: (1) does the hydrolytic activity of small intestine enzymes decrease during hibernation? (2) Is there a correlation between changes at the enzymatic level (if they occur) and histological features of the small intestine? (3) Which are the organs more affected in size during short- and long-term fasting? (4) Is there is a metabolic depression during hibernation, beyond that expected from temperature reduction alone? (5) If a metabolic depression does occur, is there a correlation between metabolic rate changes and the size of energetically expensive organs? As was recently pointed out by Tattersall and Ultsch (Tattersall and Ultsch, 2008) '[t]he crucial question in terms of understanding the biology of overwintering then becomes one of how a frog stays alive by balancing its energy supply and demand...'. It is our expectation that the present study will provide some answers to this question.

MATERIALS AND METHODS

Species biology and experimental design

The Andean toad, *B. spinulosus*, is a medium-sized, omnivorous toad that mainly feeds on small arthropods (Naya et al., 2009a). *Bufo spinulosus* is a thigmothermic species; body temperature of individuals in the field during the activity season ranges from 12°C to 32°C (Pearson and Bradford, 1976; Sinsch, 1989). At our study site (near the locality of Farellones, Chile, 33 deg.21′34″ S, 70 deg.17′57″ W, 2340 m above sea level), animals are active during spring and summer months (i.e. October–March) with activity decreasing by early autumn (April). During winter months (June–September), the habitat is covered by large amounts of snow and individuals hibernate in subnivean habitats. Toads emerge from hibernation at the beginning of spring (late September–early October). Although there is no information for *B. spinulosus*, data for other bufonids suggest that these species are not freeze-tolerant and thus must avoid microclimates below their supercooling points (Storey and Storey, 1986).

Six adult specimens of *B. spinulosus* were collected by hand from under stones placed below the snow cover in September 2007 (i.e. at the end of the Austral winter), and 16 adult specimens were collected from marginal areas of ephemeral water bodies during January 2008 (at the beginning of the Austral summer). Animals captured during summer were randomly assigned to one of two groups: toads in the first group were killed the day after their collection (as was also the case for winter animals) whereas toads in the other group were killed after two weeks of fasting. Thus, our experimental design used three groups: hibernating (N=6), fasting (N=8) and feeding (N=8) animals.

In the laboratory, animals were housed in individual cages (150×300×200 mm) covered with soil, and kept in a room at an ambient temperature of 4±1°C (in winter) or 20±1°C (in summer) under natural photoperiod. Water was added to a cotton swab placed at the bottom of the cages so that the toads could avoid dehydration. For each individual, body mass (m_b) and snout–vent length (SVL) were measured with an electronic balance (±0.1 g; Sartorious, Göttingen, Germany) and a digital calliper (±0.01 mm; Mitutoyo, Aurora, IL, USA), respectively.

All of the research conducted as part of this study conformed to national and institutional guidelines for research on live animals (SAG permit No. 4751).

Standard metabolic rates

The metabolic rate of each individual was measured the day after collection (in the hibernating and feeding groups) or after two weeks of fasting (in the fasting group). CO_2 production was measured in a Datacan V open-flow respirometry system (Sable Systems, Henderson, NV, USA) in a metabolic chamber of 0.5 l at ambient temperatures of 5°C and 15°C. To avoid any potential bias due to a setup acclimation effect, half of the animals in each group were first measured at 5°C and the other half of the animals were first measured at 15°C. The metabolic chamber received dried air at a rate of 200 ml min^{-1} from mass flow controllers (Sable Systems), and the air was passed through CO_2 absorbent granules (Baralyme®, St Louis, MO, USA) before entering the chamber. CO_2 production was monitored two times per second for one hour. Each record was automatically transformed and recorded in ExpeData® software (Sable Systems). The standard metabolic rate (SMR) was estimated as the continuous range of the lowest 3-min samples recorded during the period of data collection. Animals stayed in the chamber for five minutes before the measurements began and their activity during the recording period was determined directly by visual observation. Before and after each measurement, m_b was recorded in an electronic balance (±0.1 g) (Sartorious, Göttingen, Germany).

Morphological determinations

Toads were cooled by decreasing the ambient temperature (0°C), and they were then killed by decapitation. Animals were then

dissected and their stomach, small and large intestine, liver, heart, kidneys, abdominal fat bodies and gonads were removed. Stomach and intestines were aligned along a ruler and measured to the nearest mm. The small intestine was washed with a 0.9% NaCl solution, carefully dried with paper towels and weighed (±0.0001 g; Chyo, Kyoto, Japan). Two 2-cm sections from the anterior portion of the small intestine were obtained from all individuals: one portion was immediately frozen in liquid nitrogen for enzyme activity determinations and the other was fixed in 10% formalin for histological analysis (see below). All of the remaining organs were washed with 0.9% NaCl solution, dried in an oven at 60°C for two weeks (together with the animal's carcass) and weighed individually (±0.0001 g) (Chyo, Kyoto, Japan).

Enzyme activity measurements

We analysed disaccharidases (maltase and trehalase) and aminopeptidase-N as indicators of digestive capacity of carbohydrates and proteins, respectively (Vonk and Western, 1984). Trehalase and maltase hydrolyse the disaccharides trehalose and maltose, both yielding two molecules of glucose that can then be absorbed by the small intestine (Vonk and Western, 1984). Aminopeptidase-N cleaves NH2-terminal amino acid residues from luminal oligopeptides to produce dipeptides and amino acids absorbable by the small intestine (Ahnen et al., 1982). Small intestine tissues were thawed and homogenised for 30 s in an Ultra Turrax T25 homogenizer (Janke and Kunkel, Breisgau, Germany) at maximum setting, in 20 volumes of 0.9% NaCl solution. Disaccharidase activity was determined according to the method described by Martinez del Rio et al. (Martinez del Rio et al., 1995). Briefly, 35 µl of tissue homogenates were incubated at 25°C with 35 µl of 56 mmol l^{-1} sugar solutions (maltase or trehalase) in 0.1 mol l^{-1} maleate/NaOH buffer, pH 6.5. After 5 min of incubation, reactions were stopped by adding 3 ml of a stop/develop Glucose-Trinder reagent [one bottle of GOD-PAD reagent (Valtek, Chile) in 250 ml of 0.1 mol l^{-1} TRIS/HCl, pH 7 and 250 ml of 0.5 mol l^{-1} NaH_2PO_4, pH 7]. Absorbance was measured at 505 nm with a Spectronic 21D spectrophotometer (Bausch and Lomb, Rochester, NY, USA) after 18 min at 20°C. Glucose standards (0–0.5 ug ul^{-1} in 70 µl of 0.1 mol l^{-1} sodium maleate buffer, pH 6.5) were also reacted with the Glucose-Trinder reagent. Based on a standard curve for glucose, we calculated maltose and sucrose activity in µmol hydrolysed per minute per gram of wet tissue. Aminopeptidase-N assays were undertaken using L-alanine-p-nitroanilide as a substrate. Briefly, 50 µl of homogenate was diluted with 50 µl of 0.9% NaCl solution and mixed with 1 ml of assay mix (2.04 mmol l^{-1} L-alanine-p-nitroanilide in 0.2 mol l^{-1} NaH_2PO_4/Na_2HPO_4, pH 7). The reaction was incubated at 25°C and stopped after 10 min with 3 ml of ice-cold 2 mol l^{-1} acetic acid. We calculated the liberated amount of p-nitroanilide from the absorbance at 384 nm, then we estimated the aminopeptidase-N activity by using a standard curve constructed for p-nitroanilide. Enzyme activities were quantified as µmoles of substrate liberated per minute of incubation per gram of intestinal wet tissue (µmol min^{-1} g^{-1}).

Histological determinations

In four individuals of each group, the small intestine tissue previously fixed in 10% formalin was washed in running water, dehydrated through an increasing ethanol series and embedded in paraffin wax, according to common optical microscopy techniques (Bancroft and Stevens, 1996). Eight to 12 tissue samples of 4 µm thick were sliced from each animal, set on slides and stained using hematoxylin and eosin. Images were taken with a Nikon TE 300 light microscope (Nikon, Tokyo, Japan) with a mounted CoolSnap-Prof camera (Meyer Instruments, Houston, TX, USA). Images were captured digitally and subsequently downloaded into the software Image Pro® (Media Cybernetics, Silver Spring, MD, USA), which was used to take measurements using line morphometry. For each specimen we measured: (1) the intestinal diameter; (2) the width of the muscularis externa, which includes the circular and longitudinal muscle layers; (3) the height of five randomly selected villi, taken from the innermost edge of the circular smooth muscle layer to the outermost edge of the mucosal layer; and (4) the height and diameter of five randomly selected enterocytes at the tip of the villi. The first two histological variables were measured from two different slides for each animal whereas the last two variables were measured from one slide for each animal.

Statistical analysis

Differences among groups in body size (m_b and SVL) and enzyme standardised activities were analysed by one-way analyses of variance (ANOVAs). Changes in digestive organ length, internal organ mass and SMR were evaluated by one-way analyses of covariance (ANCOVAs), using SVL (for organ length), carcass dry mass (for organ mass) or m_b (for SMR) as covariates. In addition, to reach a better understanding of the pattern of variation in non-reproductive organ masses, a factor analysis was conducted. The effect of organ dry mass on SMR was evaluated through Pearson product–moment correlation coefficients. To remove the effect of body size on both variables, we used the residuals of the relationships between SMR and m_b and the residuals of the relationships between the mass of each organ and the carcass dry mass. Differences in histological measurements were analysed through repeated-measures one-way ANOVAs. Prior to each statistical analysis, data were examined for assumptions of normality and homogeneity of variance, using Kolmogorov–Smirnov and Levene tests, respectively. In some cases, data were transformed to the logarithm (e.g. small intestine length, enzyme activities) or to the inverse (liver dry mass) to meet the assumptions of the analyses. Interactions between covariates and factors were tested by a parallelism test, and separate-slope model was used when necessary (liver dry mass). Differences among groups were evaluated by Tukey (unequal N) HSD tests. Statistical significance was established at the 0.05 level. All the analyses were performed using the statistical package Statistica® (Statsoft, Tulsa, OK, USA) version 6.0 for the Windows® (Microsoft Corporation, Redmond, WA, USA) operating system.

RESULTS

Body and organs size

There was no difference in m_b or SVL among groups (Table 1). Except for the large intestine, which did not differ among groups, digestive organ length and mass were both greater in feeding than in fasting toads and in fasting compared with hibernating animals (Table 1; Fig. 1). Liver dry mass also changed gradually between feeding, fasting and hibernating animals but in this case differences between fasting animals and the other two groups did not reach statistical significance (Table 1). Abdominal fat bodies were heavier in both summer groups than in hibernating toads whereas kidneys were heavier in feeding animals than in both fasting groups (Table 1). No differences among groups were found for dry mass of heart and lungs (Table 1). Thus, non-reproductive organs may be grouped into those that were affected by both season and feeding condition (small intestine, stomach and liver), those that were only affected by season (fat bodies), those that were only affected by feeding condition (kidneys) and, finally, those that did not change

Table 1. Body mass (m_b), snout–vent length (SVL), digestive organ length, and organ dry mass for each group

	Hibernating	Fasting	Feeding	*F* and *P* values
Body size				
m_b (g)	77.9 (±5.3)	66.3 (±5.0)	68.7 (±8.0)	$F_{2,19}$=0.82; P<0.46
SVL (cm)	9.225 (±0.309)	8.558 (±0.252)	8.999 (±0.333)	$F_{2,19}$=1.31; P<0.29
Gut length (cm)				
Stomach	2.6 (±0.2)[a]	3.7 (±0.2)[b]	4.2 (±0.2)[c]	$F_{2,18}$=24.8; P<0.0001
Small intestine	9.8 (±1.2)[a]	14.7 (±1.1)[b]	18.9 (±1.0)[c]	$F_{2,18}$=22.7; P<0.0001
Large intestine	2.4 (±0.3)	2.4 (±0.2)	2.8 (±0.2)	$F_{2,18}$=1.18; P<0.33
Dry mass (mg)				
Stomach	0.128 (±0.020)[a]	0.227 (±0.017)[b]	0.310 (±0.017)[c]	$F_{2,18}$=24.3; P<0.0001
Large intestine	0.054 (±0.011)	0.038 (±0.010)	0.057 (±0.010)	$F_{2,18}$=1.08; P<0.37
Liver	0.576 (±0.074)[a]	0.728 (±0.057)[a,b]	0.847 (±0.055)[b]	$F_{2,16}$=3.74; P<0.001
Kidneys	0.084 (±0.007)[a]	0.087 (±0.006)[a]	0.111 (±0.006)[b]	$F_{2,18}$=5.51; P<0.02
Heart	0.067 (±0.005)	0.084 (±0.005)	0.076 (±0.005)	$F_{2,18}$=2.62; P<0.10
Lungs	0.108 (±0.011)	0.093 (±0.010)	0.111 (±0.009)	$F_{2,18}$=1.00; P<0.39
Fat bodies	0.947 (±0.582)[a]	2.833 (±0.495)[b]	3.278 (±0.497)[b]	$F_{2,18}$=4.83; P<0.03
Gonads (males)	0.019 (±0.018)	0.020 (±0.014)	0.031 (±0.015)	$F_{2,6}$=0.18; P<0.84
Gonads (females)	0.538 (±0.176)	0.205 (±0.140)	0.179 (±0.159)	$F_{2,8}$=0.37; P<0.71

Values presented are absolute means ± 1 s.e.m. (for m_b and SVL) and adjusted means ± 1 s.e.m. (for the remaining variables; covariates: SVL=8.934 cm, carcass dry mass=13.2 g). Different letters indicate significant differences (P<0.05) after a Tukey HSD test.

between groups (large intestine, heart and lungs) (Fig. 2). No differences among groups were found for dry mass of gonads; however, at least for females this is probably related to the small sample size, because values observed during winter were more than 2.5 times greater than those observed in summer.

Digestive enzymes and histology

Activities of both disaccharidases were highest in feeding animals, followed by fasting specimens and then by hibernating toads. Activity of aminopeptidase-N was also higher in feeding than in hibernating toads but in this case fasting animals did not differ from the other two groups (Fig. 3A). When enzymatic activity was integrated over the entire small intestine (i.e. standardised activity times small intestine wet mass) differences among the three groups for disaccharidase activities were even more noticeable whereas aminopeptidase-N showed greater values in feeding animals than in the other two groups, which were not statistically different (Fig. 3B).

Given that observed histological changes during fasting qualitatively agreed with recent descriptions for other amphibian species (see Discussion), here we only describe quantitative changes. We found that small intestine diameter, villus height and enterocyte height increased from hibernating to fasting toads and this increase was even more noticeable between fasting and feeding animals (Table 2; Fig. 4). By contrast, width of muscularis externa and enterocyte diameter did not change among the three groups (Table 2).

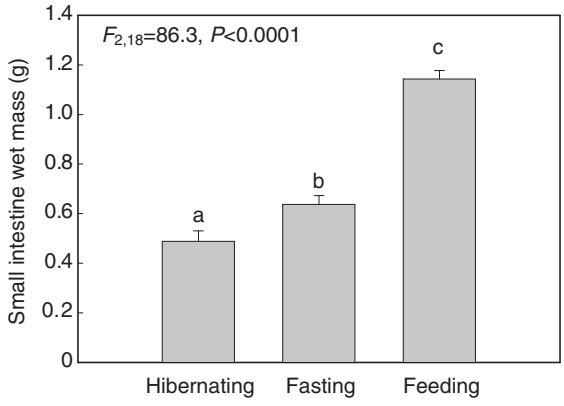

Fig. 1. Small intestine wet mass for each group. Values presented are adjusted mean values +1 s.e.m. (covariate: carcass dry mass=13.2 g). Different letters indicate significant differences (P<0.05) after a Tukey HSD test.

Standard metabolic rate

SMR was markedly higher in both summer groups than in hibernating animals. Specifically, SMR during hibernation dropped to 7.2–8.6% at 5°C and to 13.6–17.6% at 15°C of the values recorded in summer (Fig. 5A). Regarding the relationship between (residuals of) organ dry masses and (residuals of) SMR, a significant, positive correlation was observed for the stomach, small intestine, liver and fat bodies and, to a lesser extent, for the heart and kidneys (Table 3). In addition, when the mass of all internal organs was summed and its residuals were plotted against the residuals of SMR, a strong positive relationship between both variables was observed (Fig. 5B). Finally, temperature sensitivity (Q_{10}) of SMR also differed among groups ($F_{2,18}$=7.53, P<0.005), being larger in hibernating (3.25±0.51) than in fasting (1.65±0.48) and feeding animals (0.62±0.45).

DISCUSSION

The vertebrate class Amphibia have persisted through eons of environmental challenges (Feder, 1992), and under the current environmental scenario, amphibians are the ectothermic tetrapods that inhabit the highest latitudes (Darlington, 1957). Much of these extensive ranges of temporal existence and spatial distribution is related to their ability to reduce activity during long periods with unfavourable conditions and then to condense their activity into brief favourable periods (Pinder et al., 1992). In turn, as we will discuss, this ability is connected with a great flexibility at different organisational levels, which determine changes in both energy acquisition and expenditure process along the seasonal cycles.

Changes in digestive features

Both short- and long-term fasting in *B. spinulosus* is accompanied by several digestive adjustments, at least at three different levels. Firstly, fasting individuals showed lower hydrolytic activities than

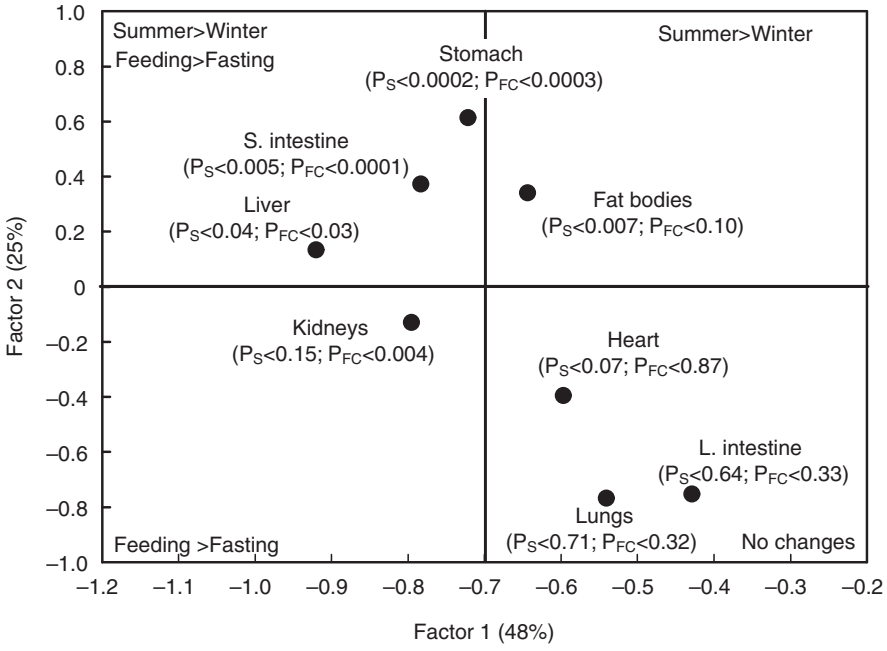

Fig. 2. Graphical representation of the factor analysis for non-reproductive organ mass. *P*-values of one-way analyses of co-variance (ANCOVAs) with season (S) or feeding condition (FC) as the main factor are provided between brackets (in all cases d.f.=19). Total amount of variance explained by each factor is given in the graph axes.

feeding animals for all three digestive enzymes analysed. This result agrees with recent studies conducted in amphibians and reptiles, which found an important decrease in nutrient uptake rates during fasting periods (Secor, 2001; Secor, 2005) and also with data on digestive enzymes during fasting in reptiles (Christel et al., 2007; Cox and Secor, 2008; Naya et al., 2009b; Ott and Secor, 2007). An interesting point regarding the observed variation in *B. spinulosus* is that hydrolytic down-regulation from feeding to long-term fasting animals was markedly lower for aminopeptidase-N (two times) than for maltose (16 times) and trehalose (six times). We suspect that this difference could be related to the re-absorption of endogenous proteins from the small intestine during fasting periods.

Secondly, there are several histological differences between feeding and fasting individuals. Specifically, we found that mucosa epithelium of feeding animals was a single-layered columnar epithelium with an observable brush border. In addition, the cytoplasm of the enterocytes was filled with lipid droplets, the nuclei were positioned basally or medially, and paracellular spaces were observed between the basal parts of the enterocytes. By contrast, mucosa epithelium of short-term fasting animals has the appearance of a pseudostratified epithelium, i.e. nuclei and cytoplasmic bodies of the enterocytes were arranged in several layers whereas their bases were attached to the basal membrane. Enterocytes were of small size, contained no lipid droplets, brush border was hardly visible and no paracellular spaces were found. All these changes are in accordance with current descriptions for other ectothermic species (Cramp and Franklin, 2005; Lignot et al., 2005; Starck and Beese, 2001; Starck and Beese, 2002; Starck et al., 2007) and support the mechanism of up- and down-regulation during the feeding/fasting cycle recently proposed for ectothermic vertebrates (Starck et al., 2007). During hibernation, enterocytes contained no lipid droplets and were even smaller than during fasting; interestingly, intestinal mucosa appears to be a single-layered (as observed in feeding animals) more than a pseudostratified epithelium (as observed in fasting animals).

Thirdly, we found a noticeable change in digestive organ gross morphology. Both length and mass were greatly reduced during short- and long-term fasting. A similar pattern of response has been reported for several other vertebrate species that are able to spend long periods of time without food consumption [amphibians (Cramp and Franklin, 2003; Naya and Bozinovic, 2004; Secor, 2005); lizards (Naya et al., 2008b; Naya et al., 2009b; Starck et al., 2007; Tracy and Diamond, 2005); rodents (Carey, 2005; Ferraris and Carey, 2000; Hume et al., 2002)]. In addition, down-regulation values for the Andean toad quantitatively agree with previous findings for other anuran species. After two weeks of fasting, we observed a decrease in small intestine length to 77.8% and a decrease in wet mass to 55.8%, in relation to animals that were feeding. These results are very close to the mean down-regulation values reported for six other anuran species after the same fasting period (length: 72.3%, wet mass: 48.6%) (Secor, 2005). Regarding long-term fasting effects, data are available for only one anuran species, the green-striped burrowing frog (*Cyclorana albogutata*). The observed small intestine length

Table 2. Small intestine histological measurements for each group

	Hibernating (*N*=4)	Fasting (*N*=4)	Feeding(*N*=4)	*F* and *P* values
Intestinal diameter (mm)	1.79 (±0.13)[a]	2.44 (±0.11)[a]	3.66 (±0.33)[b]	$F_{4,16}$=5.52; *P*<0.01
Muscularis externa width (μm)	81.62 (±5.43)	78.50 (±4.55)	61.82 (±12.22)	$F_{4,16}$=1.09; *P*<0.40
Villus height (μm)	501.1 (±48.2)[a]	643.2 (±31.4)[a]	1024.5 (±93.9)[b]	$F_{10,10}$=3.03; *P*<0.05
Enterocyte height (μm)	39.7 (±3.4)[a]	62.2 (±2.4)[b]	78.9 (±6.6)[c]	$F_{10,10}$=4.69; *P*<0.05
Enterocyte diameter (μm)	11.0 (±0.1)	10.7 (±0.8)	11.5 (±0.4)	$F_{10,10}$=0.83; *P*<0.62

Values presented are absolute means ± 1 s.e.m. Different letters indicate significant differences (*P*<0.05) after a Tukey HSD test.

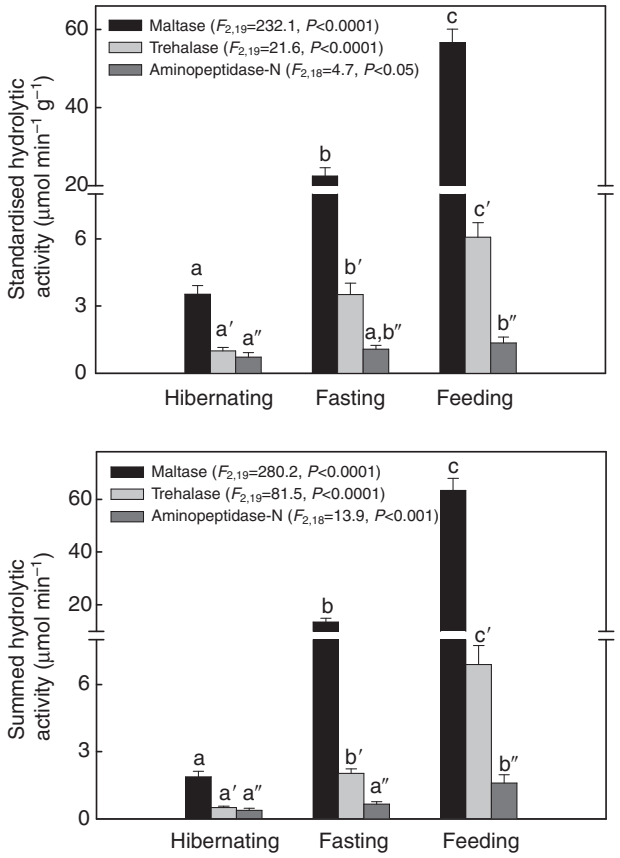

Fig. 3. (A) Digestive enzyme hydrolytic activity for each group, expressed as standardised activity per gram of small intestine wet mass tissue, and (B) integrated along the entire small intestine. Error bars=+1 s.e.m. Different letters indicate significant differences (P<0.05) after a Tukey HSD test. Note: aminopeptidase-N activity could not be determined for one specimen in the fasting group.

reductions for *B. spinulosus* after (*ca.*) four months were similar to those reported for *C. albogutata* after three months of fasting (51.9% and 55.6%, respectively), although the reduction in wet mass appears to be greater for the latter species (42.8% and 20.6%, respectively) (Cramp and Franklin, 2003).

Changes in other internal organs

It has been proposed that heart and lung size should not change during hibernation because cardiac and pulmonary performance must be maintained during dormancy (Loveridge and Wither, 1981). This finding was recently observed for the lizard *Sauromalus obesus* (Tracy and Diamond, 2005) and is confirmed in our present study animal model but see contrasting results for *Liolaemus bellii* in Naya et al. (Naya et al., 2008b) and for *Liolaemus nigroviridis* in Naya et al. (Naya et al., 2009b). However, kidney dry mass in *B. spinulosus* was affected by feeding condition but not by season, a result that diverges from those reported for the lizard species mentioned above (in all of the three cases, greater values were observed during winter). Hence, from our point of view, scarcity of data and the disparity of observed results still preclude reaching a final conclusion on the seasonal patterns of variation of these central organs.

Seasonal changes in energy storage organs for anurans that inhabit seasonal environments have been analysed for a long time (Pinder et al., 1992). For temperate species, lipid and glycogen 'build-up' occurs largely during late summer or early autumn, and these reserves are then used during winter and spring for maintenance, gamete synthesis and other reproductive activities (Elmberg, 1991; Fitzpatrick, 1972; Lu, 2004; Pasanen and Koskela, 1974). Thus, data here reported for *B. spinulosus* appear to be coincident with this annual cycle of variation. In addition, when changes in liver and fat body dry mass are compared, the former organ was mainly affected by the feeding condition whereas the latter organ was mainly affected by the season. This reinforces the idea that the temporal response of the liver is shorter than those of the fat bodies, and that carbohydrates are mainly used at the beginning of a long-term fasting period and then animals depend on the use of their lipid reserves (Van Beurden, 1980).

On the other hand, fat body mass during summer was 4.8% of the overall body mass, representing an energy reserve of 123.25 kJ (3.278 g×37.6 kJ g^{-1}). Assuming that individuals rely on fatty acid oxidation for energy during hibernation (Guppy et al., 1994), maintenance cost can be estimated as 0.3011 kJ per day, because SMR at 5°C=10.7832 ml CO_2 day^{-1} and energy equivalent for lipids=27.92 J ml CO_2^{-1} (Hill and Wise, 1989). That means that just the energy stored in the fat bodies would allow animals to satisfy their maintenance cost for more than 400 days, a period far beyond the four or five months that hibernation lasts. Consequently, we are able to hypothesise that an important amount of energy stored in the fat bodies during the previous season could be used for reproductive activities during hibernation (e.g. gamete synthesis in females) or after hibernation but before the beginning of a new feeding activity period (e.g. calling and breeding activity in males) (Girish and Saidapur, 2000). In support of this, fat bodies at the end of the hibernation, when synthesis of egg masses occurred but calling activities had not begun, are more than two times greater in males (1.64±0.50 g, N=3) than in females (0.80±0.50 g, N=3); this difference is reduced and reversed during summer months (males: 2.42±0.62 g, N=7; females: 3.26±0.56 g, N=9).

Regarding the annual cycle of the reproductive organs, two modes of ovarian development have been proposed for temperate anuran species; species in which vitellogenetic growth of oocytes occurs during hibernation and species in which vitellogenetic growth finished before the onset of hibernation and ovarian mass remains constant during hibernation (Lu, 2004). In the present study, we found that female gonads appear to be heavier during winter than during summer but, unfortunately, the lack of data for autumn months did not allow us to know when oocyte growth occurs. However, comparison of seasonal changes in energy reserves between females and males suggests that gamete synthesis proceeds during hibernation. By contrast, we found a greater proportion of females with abdominal eggs during winter (two of three females)

Table 3. Pearson product moment correlation between residuals of organs mass and standard metabolic rate (SMR) (with respect to carcass and body mass, respectively)

	SMR at 5°C	SMR at 15°C
Stomach	R=0.64, P<0.002	R=0.76, P<0.0001
Small intestine	R=0.46, P<0.04	R=0.71, P<0.0003
Large intestine	R=0.20, P<0.39	R=0.03, P<0.89
Liver	R=0.75, P<0.0001	R=0.62, P<0.003
Kidneys	R=0.23, P<0.31	R=0.42, P<0.06
Heart	R=0.49, P<0.04	R=0.33, P<0.15
Lungs	R=0.21, P<0.37	R=0.12, P<0.62
Fat bodies	R=0.66, P<0.002	R=0.53, P<0.02
Gonads	R=0.10, P<0.68	R=0.05, P<0.82

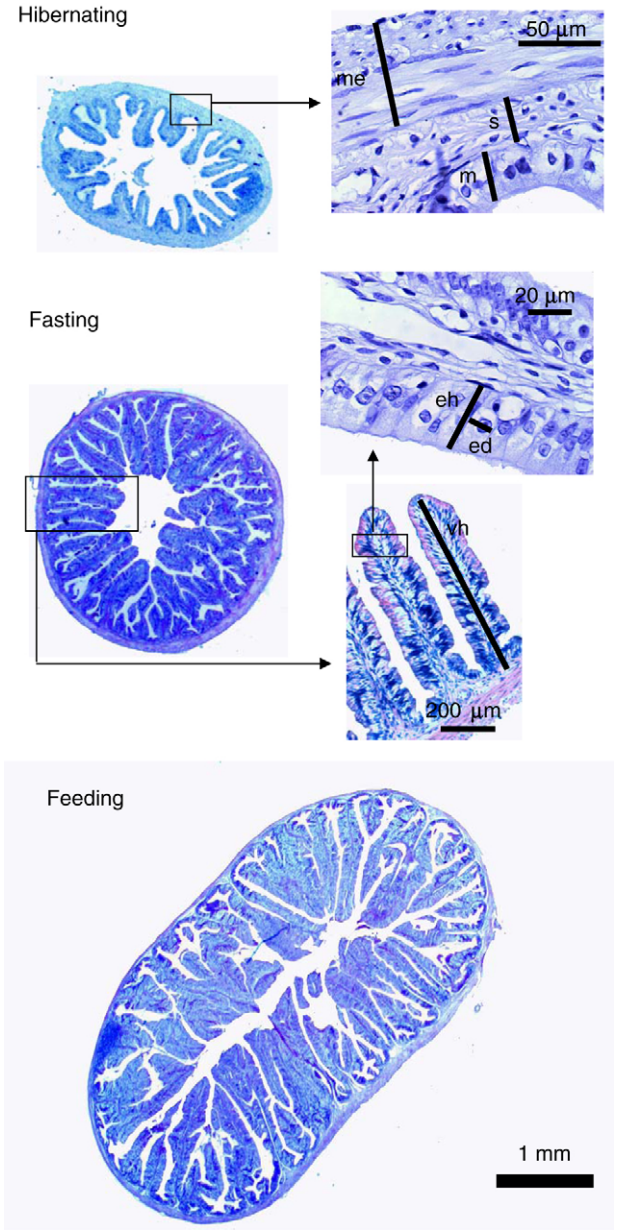

Fig. 4. Transverse sections through the proximal small intestine of a hibernating, a fasting and a feeding specimen of *Bufo spinulosus*. Abbreviations: me=muscularis externa; s=serosal layer (at the base of the villi); m=mucosal layer (at the base of the villi); vh=villus height, eh=enterocyte height; ed=enterocyte diameter.

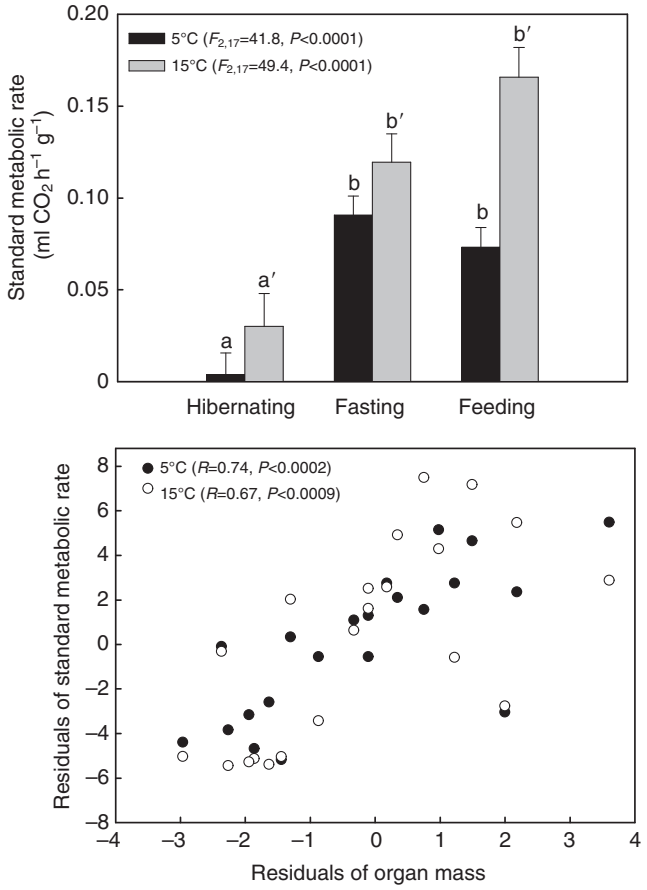

Fig. 5. (A) Standard metabolic rate (SMR) for each group determined at 5°C (black bars) and 15°C (grey bars). Values presented are adjusted means +1 s.e.m. (covariate: body mass=69.2 g). Different letters indicate significant differences (P<0.05) after a Tukey HSD test. (B) Correlation between residuals of summed organ mass and residuals of SMR at 5°C (closed circles) and 15°C (open circles). Note: the record of one animal in the feeding group did not stabilise; thus, it was discarded.

than during summer (three of nine females), confirming the fact that, at our study locality, oviposition mainly occurs during spring months (Soto et al., 2008). Interestingly, abdominal egg masses observed in the two hibernating females (4.74 g and 4.27 g) were similar to those of the summer feeding female (4.86 g) but greater than those of the two short-term fasting females (3.24 g and 2.25 g). Thus, it is possible that some re-absorption of egg material takes place during fasting (Seymour, 1973), although the idea of a small second clutch in the season cannot be discarded.

Changes in metabolic rate

In mammalian and avian physiology, hibernation implies by definition a reduction in the metabolic rate (Webster, 1974). Whether this is also the case for ectothermal animals has been a matter of some controversy. For example, Pinder et al. stated that the metabolic reduction in hibernating amphibians probably is due to lower temperatures during winter together with larger Q_{10} effect at lower temperatures and not to a different metabolic state (Pinder et al., 1992). However, a series of experiments conducted in the European water frog (*Rana temporaria*) during the last ten years clearly showed that this species can dramatically suppress its metabolic rate in response to prolonged cold-submergence, as occurs during hibernation. Specifically, it was found that submerged individuals in cold water (<5°C) suffer a metabolic depression between one-half and one-third of air breathing controls, a value that is further reduced (to one-fifth of controls) when frogs are submerged for longer periods of time (Tattersall and Ultsch, 2008). In the present study, we found a noticeable seasonal change in the metabolic rate of *B. spinulosus*: SMR of hibernating animals was only between 8.0% (at 5°C) and 13.6% (at 15°C) of summer animals. Comparing our results with previous findings, metabolic depression in the Andean toad is slightly higher than that observed in *R. temporaria* and also than those reported for most aestivating amphibians species studied to date (15–40% of non-aestivating values) (Guppy and Whiters, 1999). Moreover, the estimation of

mass-specific metabolic rate of *B. spinulosus* at 0°C, i.e. the most likely temperature for the subnivean habitat, gives a value of $0.027\,W\,kg^{-1}$, which is very close to the lower range of 'minimum life-supporting metabolic rate' observed for cold water ectothermic vertebrates (95% CI: 0.016–$0.23\,W\,kg^{-1}$) and aestivating ectothermic vertebrates (95% CI: 0.022–$0.27\,W\,kg^{-1}$) (Makarieva et al., 2006).

Finally, two additional points regarding seasonal changes in *B. spinulosus* metabolic rate should be mentioned. Firstly, there is a clear correlation between organ mass and SMR, suggesting that a reduction in the size of energetically expensive organs, such as kidneys, heart, liver, and gut (Guppy et al., 1994; Morris, 1980; Penick et al., 1996), could be a major explanation for the metabolic depression observed during hibernation. Interestingly, this potential ability to modulate SMR through seasonal change in organ size is not usually mentioned in current studies on amphibian hibernation (e.g. Storey and Storey, 2007; Tattersall and Ultsch, 2008). Secondly, we found that thermal sensitivity of the metabolic rates was three times higher at the end of the hibernation than in the middle of summer. Seasonal changes in Q_{10} values have been reported for other hibernating anurans and are considered an adaptation to a seasonal environment, because they allow the rapid recovery of animals after a long period of quiescence (Dunlap, 1980).

General conclusion

How efficiently organisms use their finite energy stores during overwintering starvation is of fundamental relevance to their survival and lifetime fitness (Boutilier et al., 1999). In the present study we showed that: (1) *Bufo spinulosus* individuals are able to strongly reduce their energy fluxes during winter months; (2) this ability implies several phenotypic adjustments at different biological organisation levels (e.g. biochemical, structural, physiological); (3) the magnitude of these phenotypic changes is similar to (or even greater than) those usually observed for amphibian species that hibernate submerged under water or aestivate buried in the soil. We think that, given the pressing need to explain and predict the impact of climate change on the biota, more studies comparing different physiological strategies and determining the limits of structural and physiological flexibility are necessary.

LIST OF ABREVIATIONS

ANCOVA	analysis of co-variance
ANOVA	analysis of variance
CI	confidence interval
d.f.	degrees of freedom
m_b	body mass
s.e.m.	standard error of the mean
SMR	standard metabolic rate
SVL	snout–vent length

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