Metabolic responses of the South American ornate horned frog (*Ceratophrys ornata*) to estivation

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ABSTRACT

We examined the metabolic responses of the South American frog, *Ceratophrys ornata*, to laboratory-induced estivation. Whole-animal and mass-specific oxygen consumption rates (VO_2) did not change during fasting or 56 days of estivation, despite observing significant decreases in body mass. The maintenance of mass-specific metabolic rate at routine levels during estivation suggests that metabolic rate suppression is not a major response to estivation in this species. There was a significant decline in liver glycogen and a loss of adipose tissue mass during estivation, suggesting that both carbohydrate and lipid pathways are used to fuel metabolism during estivation. The activity of pyruvate dehydrogenase, an important regulator of carbohydrate oxidation, and carnitine palmitoyltransferase and 3-hydroxyacyl-CoA dehydrogenase, regulators of lipid oxidation, showed no significant change in activity in liver, heart, and muscle between estivating and active frogs. There was an increase in plasma osmolality, which is characteristic of estivating animals. Overall, our metabolic analysis of estivation in *C. ornata* indicates that this species does not employ a dramatic suppression metabolic rate to survive dehydration stress and that both endogenous carbohydrates and lipids are used as metabolic fuels.

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1. Introduction

Estivation is a state of dormancy characterized by a suite of morphological, physiological, biochemical and behavioral changes that enhance survival during periods of drought or low food availability. In anurans that inhabit semi-arid and arid regions, estivation occurs in response to a drying environment and can last for periods ranging from weeks to years depending on the species (Cartledge et al., 2006; Carvalho et al., 2010). Estivating anurans are quiescent and most often found buried in the substrate and, depending on the species, may be encased in a "parchment-like" cocoon to conserve water (McClanahan et al., 1976). A suppression of whole-animal oxygen consumption rate (VO_2) is also a typical response to estivation in anurans (Carvalho et al., 2010), although the degree of suppression of VO_2 varies among species. For example, frogs from the genus *Neobatrachus* can reduce whole-animal VO_2 by up to 85% below resting measurements (Withers, 1993) while other species of frog (e.g. frogs from the genus *Ceratophrys*) only suppress VO_2 by a comparatively small amount (<25% decrease in VO_2; Secor, 2005). Low metabolic rates during estivation are clearly advantageous because it extends the duration of dormancy that can be supported by endogenous fuels. In frogs that do not show a suppression of metabolic rate during estivation, they are likely to either have inherently low routine metabolic rates, larger endogenous fuel reserves, or perform shorter periods of estivation.

During estivation, anurans and other estivating animals (e.g. lungfish; *Protopterus dolloi*) shift fuel use away from carbohydrates and toward lipid and amino acid catabolism (Jones, 1980; Cowan et al., 2000; Frick et al., 2008a,b). Amino acid catabolism can support metabolic energy production, but during estivation it is generally thought that the amino acids liberated from protein breakdown are used as compatible solutes for osmoregulation as well as substrates for gluconeogenesis for use in select tissues such as the brain (Storey, 2000, 2002). Furthermore, the nitrogen released during amino acid catabolism can be incorporated into urea, which is accumulated to high concentrations in some species of frogs during estivation, such as *Neobatrachus* spp. and *Cyclorana* spp. (Withers and Guppy, 1996). As a result, it is generally accepted that lipids are the main oxidizable fuel used during estivation, which results in large depletions of endogenous lipid reserves. Most of the depleted lipids are from large triacylglycerol deposits in abdominal fat bodies (~3% of body mass in estivating frogs; Seymour, 1973a) as well as lipids stored in the liver and other organs (Pinder et al., 1992). In *Pyxicephalus adspersus*, Loveridge and Withers (1981) calculated that 3% body mass of fat could support 260 days of estivation, but this species is well known to suppress metabolic rate by up to 78%. However, in the spadefoot toad (*Scaphiopus multiplicatus*) 9 months of estivation resulted in a 51% decrease in total energy reserves of which lipids were depleted by 62%, carbohydrates were reduced by 64% and protein was depleted by 32% (Jones, 1980).

The biochemical regulation of metabolic fuel selection has not been thoroughly investigated in estivating anurans, particularly those species

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that do not suppress metabolic rate to a great extent during estivation. Fuel selection in most tissues is regulated, to a large degree, by the reversible phosphorylation of metabolic enzymes (Cowan et al., 2000). One such enzyme is pyruvate dehydrogenase (PDH), which is an important regulator of glucose oxidation. PDH catalyzes the conversion of glycolytically derived pyruvate into acetyl-CoA for entry in the tricarboxylic acid cycle and is profoundly influenced by a variety of factors related to fatty acid oxidation. An activation of lipid oxidation results in an accumulation of acetyl-CoA, mitochondrial NADH, and citrate, which inactivates PDH preventing glucose from being oxidized by the mitochondria. During periods of high fat oxidation, PDH is inhibited through phosphorylation and carbohydrate is spared (Randle, 1995). Mitochondria have also been proposed as the primary site of control of metabolic rate suppression (Bishop et al., 2002) and PDH may therefore play a critical role in initiating and regulating metabolic rate suppression during estivation.

The objective of this study was to examine the metabolic responses of the ornate horned frog (*Ceratophrys ornata*) to estivation and determine the biochemical factors that regulate fuel selection in this species. The ornate horned frog is native to South America and is known to estivate for periods up to 5 months (McClanahan et al., 1976) and recent laboratory studies have shown that 1 month of estivation in this species only induces a modest (~25%) decrease in VO$_2$ compared with fasting animals (Secor, 2005; reviewed in Carvalho et al., 2010). In order to define the metabolic responses of the ornate horned frog to estivation, we measured whole-animal VO$_2$ and body mass in groups of frogs during 14 days of fasting followed by up to 56 days of laboratory induced estivation. Tissue samples were taken from frogs after 45 to 56 days of estivation as well as from active, hydrated control frogs that were fed continuously until 16 days before sampling, when they were fasted. Tissue samples were used for analysis of regulatory enzyme activities (eg. PDH and carnitine palmitoyltransferase; CPT), enzymes involved in aerobic metabolism (citrate synthase; CS and 3-hydroxyacyl CoA dehydrogenase; HOAD), and metabolites involved in fatty acid and carbohydrate oxidation (eg. glycogen, acetyl-CoA, etc). Analysis of plasma osmolality was also performed to determine if our frogs were experiencing dehydration stress.

2. Materials and methods

2.1. Animals

Specimens of *C. ornata* were purchased from a commercial pet supplier and shipped to the University of British Columbia, Canada. Animals were housed individually in large glass aquaria with moist soil substrate, half a clay flowerpot for hiding, and a dish of water. The aquaria were held in a temperature regulated environment chamber at 25 °C and the soil was misted with dechlorinated city of Vancouver tapwater daily. The soil was replaced every week. Frogs were fed crickets, neonatal mice, or fish fillets dusted with multivitamins three times a week and feces was removed when noticed. All experiments were conducted according to the guidelines of the Canadian Council for Animal Care and protocols were approved by the University of British Columbia Animal Care Committee.

2.2. Experimental procedure

Two separate experimental series were conducted to examine the combined effects of estivation and fasting on whole-animal VO$_2$, changes in animal and organ mass, and tissue metabolic profiles. In both experimental series, frogs were fasted for 14 days followed by a roughly 56 day estivation period. To induce estivation, water was withheld and within 9 days the soil was visibly dry and frogs had assumed a water-conserving posture and cocoon formation begun, as described in other estivating frog species (Withers, 1995). In experimental series 1, a group of eight frogs (80.61 ± 1.33 g before estivation; see filled symbols in

Fig. 1F) were fasted in their holding aquarium and after 14 days the frogs were transferred to individual 400 mL glass chambers with a known weight of dried, sterilized soil (autoclaved and dried at 60 °C for three weeks; dry weight of soil was between 29.7 to 36.1 g and ~2 cm depth) to which 35 mL of dechlorinated water was added. Chambers were moved to a quiet, dark (with red light), environmentally controlled room and held open to the atmosphere at 25 °C, except during measurements of oxygen uptake rates when the chambers were sealed (see Section 2.3). Changes in body mass and VO$_2$ were monitored throughout 56 days of estivation. Frogs from experimental series 1 were not sacrificed; rather they were rehydrated and maintained as described in Section 2.1 above. In experimental series 2, a group of larger frogs (n = 8; 116.45 ± 3.78 g before fasting; see open triangles in Fig. 1E) were transferred into individual respirometry chambers before fasting was initiated and changes in VO$_2$ and body mass were monitored during the 16 day fast period as well as during the last 25 days of a 56 day estivation period (open symbols in Fig. 1F). Estivation in experimental series 2 was induced in an identical manner to experimental series 1. At the end of the estivation period, series 2 frogs were euthanized and organs (including liver, leg muscles, heart, kidneys, gonads, fat bodies and the entire length of the gut from stomach to large intestine) were dissected, cleared of their contents, weighed, and frozen in liquid N$_2$ for analysis of tissues metabolites and enzyme activities. A separate group of hydrated control frogs (referred to as active controls; n = 8; 106.56 ± 3.90 g before fasting period; see Table 1 for weight at sampling) were kept in parallel with the estivation group from experimental series 2. These active control frogs were fed daily except for a 16 day fasting period before tissue sampling and determination of organ mass.

2.3. Respirometry and mass measurements

The protocols used for the determination of VO$_2$ and mass were the same in experimental series 1 and 2. Closed respirometry was used to determine VO$_2$. A rubber stopper with a hole drilled through was used to seal the estivation chambers. A rubber septum was used to seal the hole of the stopper, and a fiber optic oxygen probe (Foxy system; Ocean Optics, Dunedin, FL, USA) housed within a cannula was inserted 2 cm into the chamber through the septum and further sealed with petroleum jelly. The partial pressure of O$_2$ was measured continuously throughout a 1.5 h respirometry period and VO$_2$ was calculated using the change in oxygen partial pressure over time, corrected for the volume of the respirometry chamber (minus the volume of the soil and the mass of the frog), temperature, atmospheric pressure, and frog mass (Vleck, 1987). Measurements were made every three to seven days. There was a 34 day window, in which respirometry measurements were not made in experimental series 2.

The mass of each frog was measured immediately after each respirometry measurement. Initial frog masses were determined on well-hydrated frogs before their placement in the estivation chamber. The changes in frog mass during estivation were calculated as the initial mass minus the change in the mass of the respirometry chamber plus frog and soil between measurements. The total mass of the respirometry chamber and its contents were determined by gently transferring the respirometry chamber to a pan balance. A pilot study determined that the initial 35 mL of water added at the beginning of the estivation trial required 9 days to completely evaporate and it was assumed that frog mass did not change during the first 9 days of estivation. Body mass of active control and fasted frogs were measured directly by transferring individual frogs from their holding aquaria to the pan balance.

2.4. Tissue sampling and organ wet mass determination

Frogs from experimental series 2 and the active control frogs were used for tissue sampling.

All estivating frogs were sampled between days 49 and 56 of estivation, except one, which was sampled at day 44 because the VO$_2$
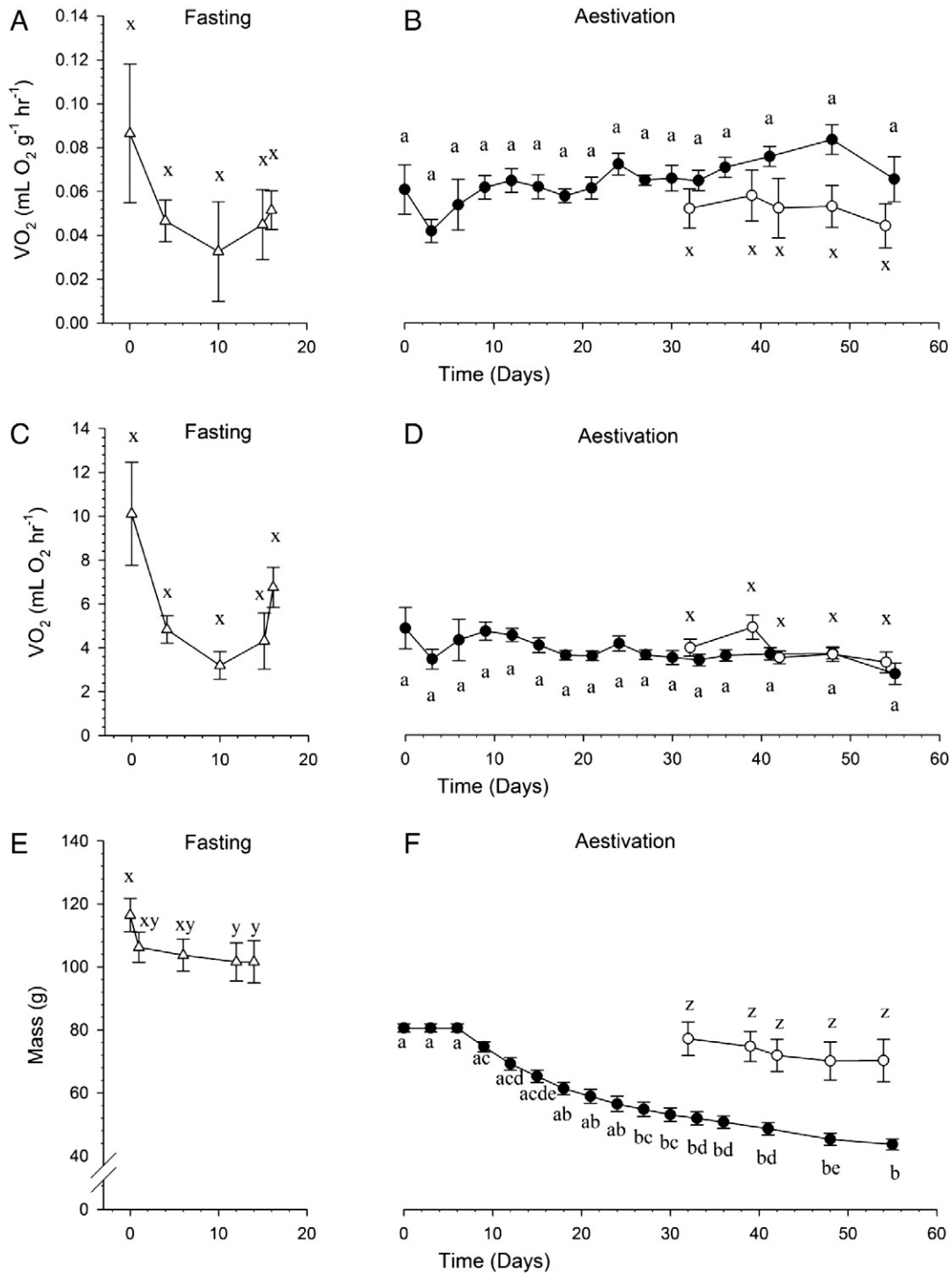
Fig. 1. Mass-specific and whole-animal oxygen consumption rates (VO$_2$) and whole-animal mass of fasting (A, C, and E) and estivating (B, D, and F) frogs, respectively. Series 1 estivating, series 2 estivating and group 2 fasting is represented by black circles, white circles and white triangles, respectively. Values are means ± SEM; n = 5–9. Values with different letters are significantly different (P<0.05). Series 1 and series 2 frogs were analyzed separately.

dropped suddenly compared to the other frogs. All frogs were sacrificed by double-pithing. Blood was sampled *via* heart puncture using heparinized syringes. Two hematocrit capillary tubes were then filled with blood and centrifuged at 2500 *g* and hematocrit was determined as the percent of red blood cells in whole blood. The remaining blood was then centrifuged at 2500 *g* and the plasma was immediately frozen and stored at −80 °C. The liver, leg muscles (including all muscles from the thigh), heart (ventricle), kidneys, gonads, fat bodies and gut (stomach through to large intestine) were quickly excised, cleared of contents when necessary, weighed to the nearest milligram, and frozen in liquid nitrogen. All samples were stored at −80 °C. Mass was expressed both in grams and percent body mass.

2.5. Plasma osmolality and metabolites

Plasma osmolality was measured using a Wescor 5500 vapour pressure osmometer (Wescor Inc., UT, USA). Non-esterified fatty acids (NEFA) were measured using a commercial kit (NEFA-HR2; Wako, Osaka, Japan). Glucose concentration was determined spectrophotometrically as described in Bergmeyer (1983).

Table 1
Whole animal and organ mass from active and estivating *Ceratophrys ornata*.

	Wet tissue mass (g)		Percent mass (%)	
	Active	Estivating	Active	Estivating
Whole animal	92.07 ± 3.25 (7)	63.89 ± 5.09* (7)		
Liver	5.23 ± 0.63 (8)	2.04 ± 0.84* (7)	5.25 ± 0.51 (7)	3.29 ± 1.39* (7)
Ventricle	0.28 ± 0.02 (6)	0.18 ± 0.01* (6)	0.31 ± 0.01 (5)	0.29 ± 0.04 (6)
Gonad	0.55 ± 0.05 (8)	0.52 ± 0.12 (6)	0.60 ± .053 (7)	0.83 ± 0.15 (5)
Fat bodies	1.70 ± 0.33 (7)	0.14 ± 0.05* (4)	1.84 ± 0.36 (6)	0.22 ± 0.09* (4)
Kidney	0.26 ± 0.03 (8)	0.17 ± 0.01* (7)	0.27 ± 0.02 (7)	0.28 ± 0.04 (7)
Gut	4.22 ± 0.31 (8)	4.63 ± 0.63 (7)	4.52 ± 0.26 (7)	7.31 ± 0.81* (7)

Values presented as mean ± SEM. Sample size indicated within the parentheses. Note that these masses are without regard to time of sampling.
  * Indicates significantly different from active (P<0.05).

2.6. Tissue enzymes and metabolites

Heart, liver and leg muscle of frogs from experimental series 2 were used for analysis of metabolite levels and enzyme activities. Tissues were ground to small pieces using a liquid nitrogen cooled mortar and pestle and stored at −80 °C. Acid soluble metabolites, including lactate, glucose, acetyl-CoA, acetyl-carnitine, and free carnitine were extracted from ~100 mg aliquots of each tissue in 1 mL of 8% $HClO_4$ with three, 10 s bursts of a Kontes sonicator set on its maximum setting. Each burst was separated by 10 s on ice. A 200 μL aliquot of the homogenate was then taken and frozen at −80 °C for determination of glycogen content. The remaining homogenate was centrifuged at 10,000 g for 10 min at 4 °C and the supernatant was neutralized using 3 M K_2CO_3 and immediately frozen at −80 °C. The neutralized extract was used for the analysis of tissue glucose and lactate concentrations using the enzymatic methods described in Bergmeyer (1983). Tissue acetyl-CoA, acetyl-carnitine and free carnitine concentration was measured on the neutralized tissue extracts as described in Richards et al. (2002). Samples for glycogen analysis were thawed on ice, partially neutralized with 3 M K_2CO_3, digested with amyloglucosidase, and measured for glucose as previously described (Bergmeyer, 1983). Glycogen is reported in units of glucosyl units/g wet mass and corrected for free glucose.

For analysis of enzyme activity, aliquots of heart, liver, and leg muscle were homogenized as described in Richards et al. (2002), using a 2 mL glass homogenizer. The activity of the active portion of pyruvate dehydrogenase (PDH; EC 1.2.4.1) was determined as previously described (Richards et al., 2002). The maximal activity of citrate synthase (CS; EC 2.3.3.1), carnitine palmitoyltransferase (CPT; EC 2.3.1.21), and 3-hydroxyacyl CoA dehydrogenase (HOAD; EC 1.1.1.35) was measured spectrophotometrically at 412 nm (CS and CPT) or 340 nm (HOAD). CS was assayed in a buffer containing (in mM) 0.30 acetyl-CoA, 0.15 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 50 Tris–HCl and 0.5 oxaloacetate. CPT was assayed in a buffer containing (in mM) 5 carnitine, 0.035 palmitoyl-CoA, 0.15 DTNB and 50 Tris–HCl. HOAD was measured in (in mM) 0.1 acetoacetyl-CoA, 0.5 NADH, 0.2 dithiothreitol and 50 Tris–HCl. Homogenate protein concentration was determined using Bradford's Reagent (Sigma-Aldrich Canada Ltd.) and all enzyme activities were expressed as μmol mg protein^{-1} min^{-1}.

2.7. Statistical analysis

For the respirometry and mass time courses, statistical analysis consisted of one-way analyses of variance (ANOVAs) followed by the Holm–Sidak post-hoc analysis to identify significant differences between time points. If the data did not fit the assumptions of a one-way ANOVA following log transformations, Kruskal–Wallis one-way ANOVA on ranks was performed followed by Dunn's post-hoc test. Repeated measures ANOVA could not be used because some animals died during

the estivation period and therefore we do not have continuous data. Also, technical difficulties prevented all animals from being measured on certain days. For comparisons of organ mass, metabolite and enzyme analysis between experimental series 2 frogs and the active controls, Student's t-tests were performed. Statistical significance was accepted at P<0.05. All data are presented as means ± SEM.

3. Results

The frogs were alert and responsive throughout the fasting period of both experimental series 1 and 2. Throughout the estivation periods, the frogs remained partially buried in the substrate with no noticeable movement. One mortality was observed during the 56 days of estivation. The frogs did not respond to their VO_2 or mass being measured.

3.1. Respirometry

In experimental series 1, up to 56 days of estivation did not significantly affect mass-specific VO_2 (Fig. 1B) or whole-animal VO_2 (Fig. 1D). In series 2, fasting and estivation also had no significant effect on mass-specific VO_2 (Fig. 1A, B) or whole-animal VO_2 (Fig. 1C, D).

3.2. Whole-animal and organ mass

In experimental series 2, fasting resulted in a decrease in body mass over the first 12 days, which remained lower than day 0 at day 14 (Fig. 1E). During the estivation period in experimental series 1, body mass remained constant for the first 9 days (while the substrate dried) followed by a gradual, 46% decrease in body mass during the 56 days of estivation (Fig. 1F). Thirty-two days of estivation resulted in a 25% decrease in body mass of frogs from experimental series 2 compared with the same frogs measured at the end of fasting (Fig. 1E). There was no change in body mass during the last 22 days of estivation in frogs from experimental series 2 (Fig. 1F). At the time of sampling, estivating frogs weighted 31% less than the active controls (Table 1).

Active control frogs had significantly larger livers (2.6 fold), heart ventricle (1.6 fold), fat bodies (12 fold), and kidney (1.5 fold) than their estivating counterparts (Table 1) except when tissue mass was expressed relative to body mass, where only the liver and fat bodies were found to be smaller in estivating frogs than the active control frogs (Table 1). Of the estivating frogs, only 4 of the 7 surviving individuals had any visible fat bodies. The total gut (including stomach and intestine) mass was not different between estivating and active control animals, but the gut contributed 1.6 fold more to the total body mass of estivating frogs than active frogs (Table 1).

3.3. Hematocrit and plasma osmolality

There was no difference in hematocrit between active and estivating frogs (Table 2). Plasma osmolality was 2 fold greater in estivating frogs compared with active frogs (Table 2).

3.4. Carbohydrate metabolism

Plasma glucose concentration was not significantly different between active and estivating frogs (Table 2). Liver glucose concentration was 4 fold higher after 56 days of estivation compared with active frogs, but there was no significant effect of estivation on heart or muscle glucose concentration (Fig. 2A). Total liver glycogen concentration of active individuals was 3.8 fold higher than estivating individuals (Fig. 2B). There was no difference in heart and muscle glycogen concentration between active and estivating frogs (Fig. 2B). Liver, heart, and muscle lactate concentration were not affected by estivation (Fig. 2C). Pyruvate dehydrogenase activities in liver, heart, and muscle were not different between active and estivating frogs (Fig. 3A).

3.5. Fatty acid metabolism

Plasma NEFA concentration was not affected by 56 days of estivation compared with the active control frogs (Table 2). CPT activity was not different between estivating and active frogs in the liver and heart (Fig. 4A). Muscle CPT activity was below detectable levels (Fig. 4A). The activity of HOAD was also not significantly different between active and estivating frogs in the liver, muscle and heart (Fig. 4B).

3.6. Acetyl-CoA, carnitine and citrate synthase activity

Liver, heart and muscle acetyl-CoA concentration did not differ between estivating and active frogs (Fig. 5A). Liver and heart acetyl-carnitine concentrations were higher in estivating animals than active frogs (~47% higher in both tissues), but there was no significant difference found within the muscle tissue (Fig. 5C). Free carnitine was higher in liver, muscle and heart of estivating frogs compared to active frogs (Fig. 5B). Liver citrate synthase activity was 2-fold higher in estivating frogs than active frogs, but there were no differences in muscle and heart citrate synthase activity between estivating and active frogs (Fig. 3B).

4. Discussion

Estivation is a period of dormancy that occurs in response to low water and food availability (Storey and Storey, 1990) and is often associated with a suppression of metabolic rate. In our hands, however, upwards of 56 days of estivation had no significant effect on whole-animal or mass specific VO_2 in the ornate horned frog, although there was a 40% decrease in body mass (Fig. 1). In spite of the lack of metabolic rate suppression, the vast majority of frogs survived the estivation period. Among different species of anurans, there is variation in the degree of metabolic rate suppression with some species experiencing dramatic decreases in VO_2 during estivation, while in other species the
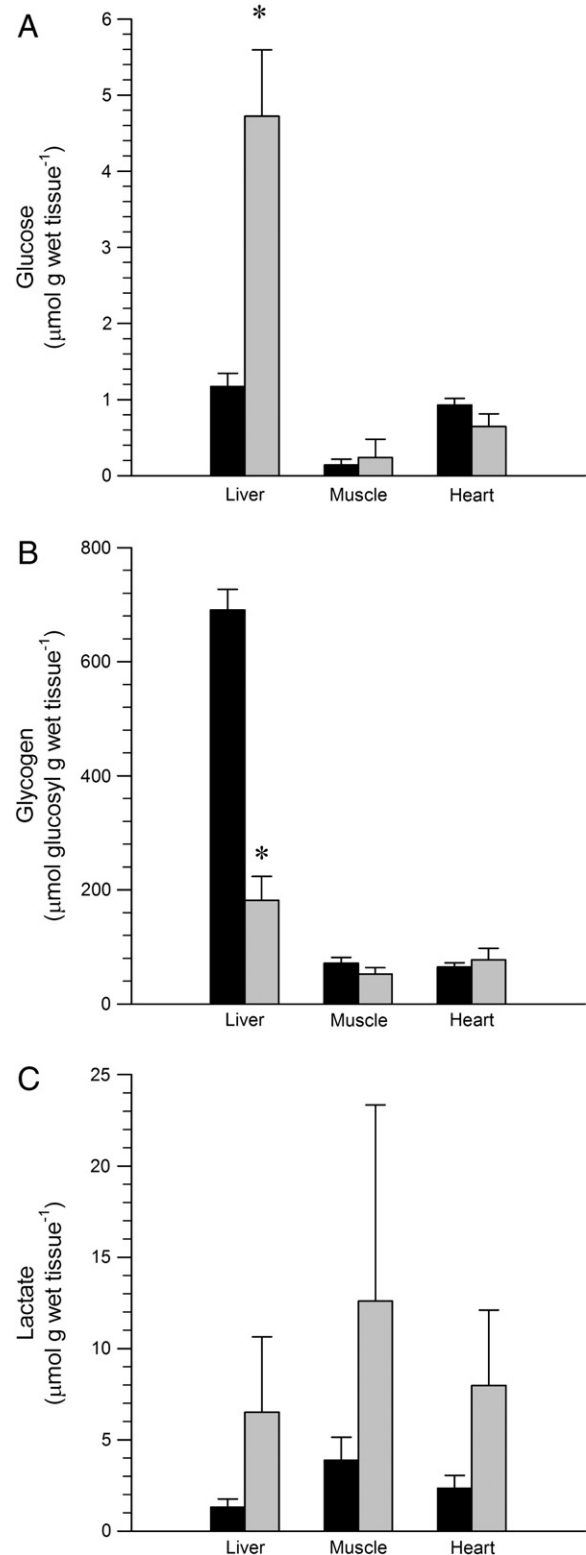

Fig. 2. Liver, muscle and heart glucose (A), glycogen (B), and lactate (C) concentrations of active (black bars) and estivating (grey bars) frogs. Values are means ± SEM; n = 5–8. * indicates a significant difference between active and estivating frogs (P<0.05).

reductions in VO_2 associated with estivation are modest or nonexistent. The green-striped burrowing frog (*Cyclorana alboguttata*) is one species that reduces VO_2 by up to 82% during estivation (Kayes et al., 2009), while the Couch's spadefoot toad reduces VO_2 by 75–83% (Seymour, 1973a) and both of these species are well known to undergo estivation. Previous work on the ornate horned frog or its close relatives
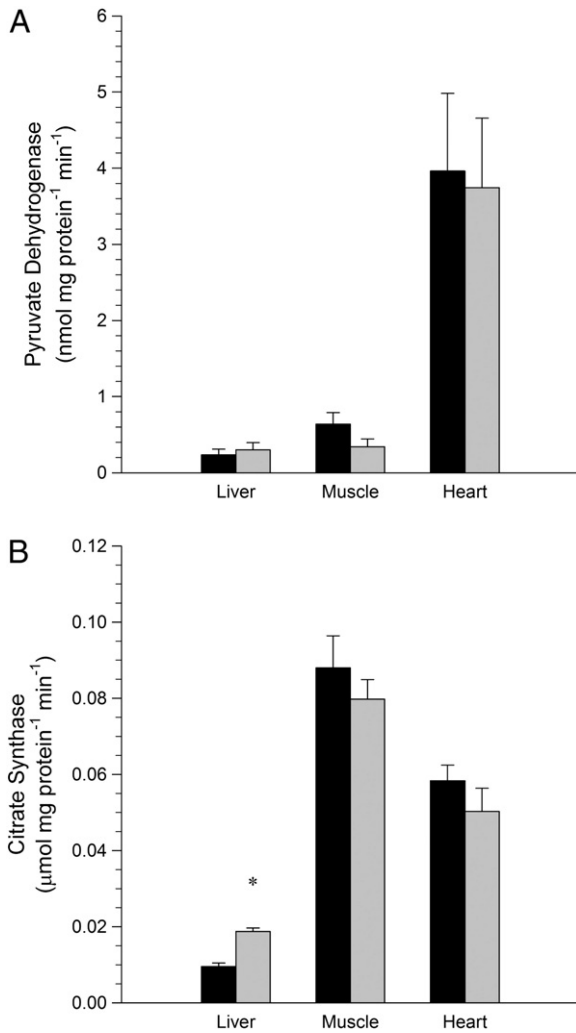
Table 2
Hematocrit, plasma osmolality and metabolite concentrations in active and estivating *Ceratophrys ornata*.

	Active	Estivating
Hematocrit (%)	28 ± 2 (5)	27 ± 2 (5)
Osmolality (mosmol/kg)	287 ± 26 (7)	601 ± 51* (6)
Osmolytes (mmol/L)		
Glucose	1.03 ± 0.11 (6)	1.04 ± 0.17 (5)
Non-esterified fatty acids	0.045 ± 0.007 (5)	0.054 ± 0.005 (5)

Values presented as mean ± SEM. Sample size indicated within the parentheses.
* Indicates significantly different from active (P<0.05).

Fig. 3. Liver, muscle and heart pyruvate dehydrogenase (A) and citrate synthase (B) activities of active (black bars) and estivating (grey bars) frogs. Values are means ± SEM; n = 5–8. * indicates a significant difference between active and estivating frogs (P<0.05).

Fig. 4. Liver, muscle and heart carnitine palmitoyltransferase (A) and 3-hydroxyacyl CoA dehydrogenase (B) activities of active (black bars) and estivating (grey bars) frogs. Values are means ± SEM; n = 4–8. * indicates a significant difference between active and estivating frogs (P<0.05).

(*Ceratophrys aurita*) indicate that members of this genus have a limited capacity to suppress metabolic rate in response to estivation and only decrease whole-animal VO$_2$ by up to 25% (Bastos and Abe, 1998; Secor, 2005), or not at all as indicated by our study. Clearly, large decreases in metabolic rate are not essential for survival of a dry, food-limited environment in this species. However, it must be noted that estivating species of frogs have been shown to have, on average, lower standard metabolic rates that non-estivating species of frogs (Secor, 2005), thus, in some species it may not be necessary to suppress metabolic rate substantially below standard levels. One other possibility for the divergent findings may be due to the length of time required to reduce metabolic rate. Individuals that are able to decrease their metabolic rate rapidly may display a lower metabolic depression than an animal that decreases over a longer timescale. Furthermore, the *C. ornata* used in the present study were captive bred animals, which may have impacted their overall ability to suppress metabolic rate compared with population that inhabit the diverse environments of South America (Canziani and Cannata, 1980). Despite the low, or non-existent suppression of metabolic rate in our frogs, the ornate horned frogs used in the present study were able to survive up to 56 days of estivation while encased in a cocoon and unresponsive to gentle prodding.

Changes in body mass can have confounding effects on the interpretation of metabolic rate suppression during fasting (see review by McCue, 2010) a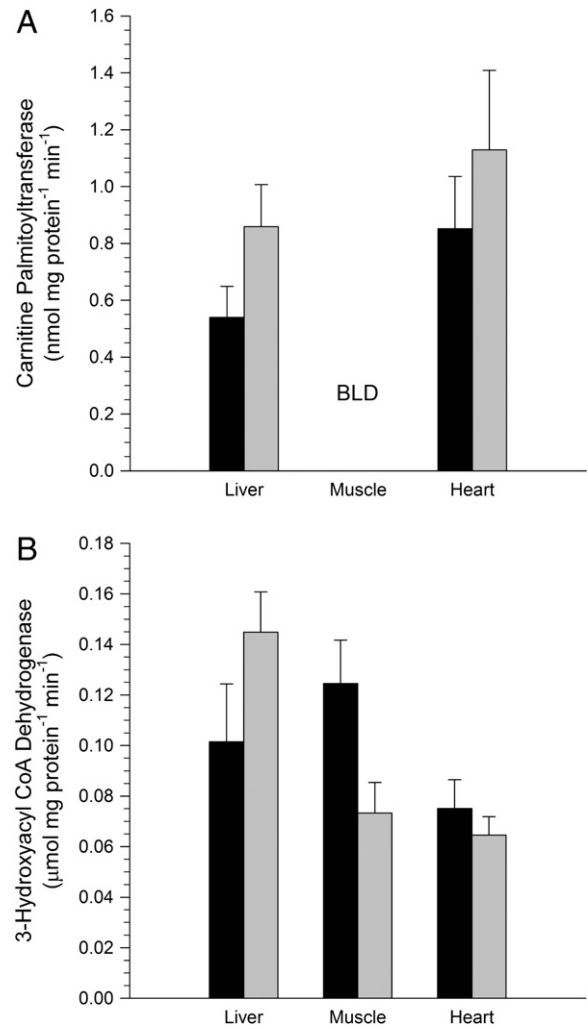nd estivation. As expected, mass of the frog declined during both fasting and estivation due to a combination of dehydration and selective atrophy of tissues. Selective atrophy occurs when only specific tissues undergo atrophy, as has been shown previously in leg muscles not used for force generation during jumping (Mantle et al., 2009). Metabolic rate measurement over time is subject to calculation constraints with concurrent changes in body mass. An effort was made to minimize this by performing both respirometric and weight measurements concurrently.

Estivating frogs lost 44% in series 1 and 30% in series 2 of body mass during estivation (Fig. 1F) and almost all organs examined, including the liver, heart ventricle, fat bodies, and kidney had lower wet weights than organs from the active, fed controls (Table 1). The loss of mass is likely accounted for by a loss of water and the atrophy of tissues due to the lack of nutrient intake during estivation. The only tissues that did not decrease in size were the gonads and total gut, which remained constant and in the case of the gut accounted for a greater percent of body mass in estivating animals than the active control animals. The small intestine is well known to undergo atrophy following periods of fasting, such as in the hibernating thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) (Carey, 1990) and other estivating frogs (Cramp and Franklin, 2005; Cramp et al., 2005), and potentially acts as an energy saving mechanism by reducing energy spent on gut function and structure (Carey, 1990; Cramp and Franklin, 2005; Cramp et al., 2005; Secor, 2005). Estivation did not affect gonad mass, suggesting that maintenance
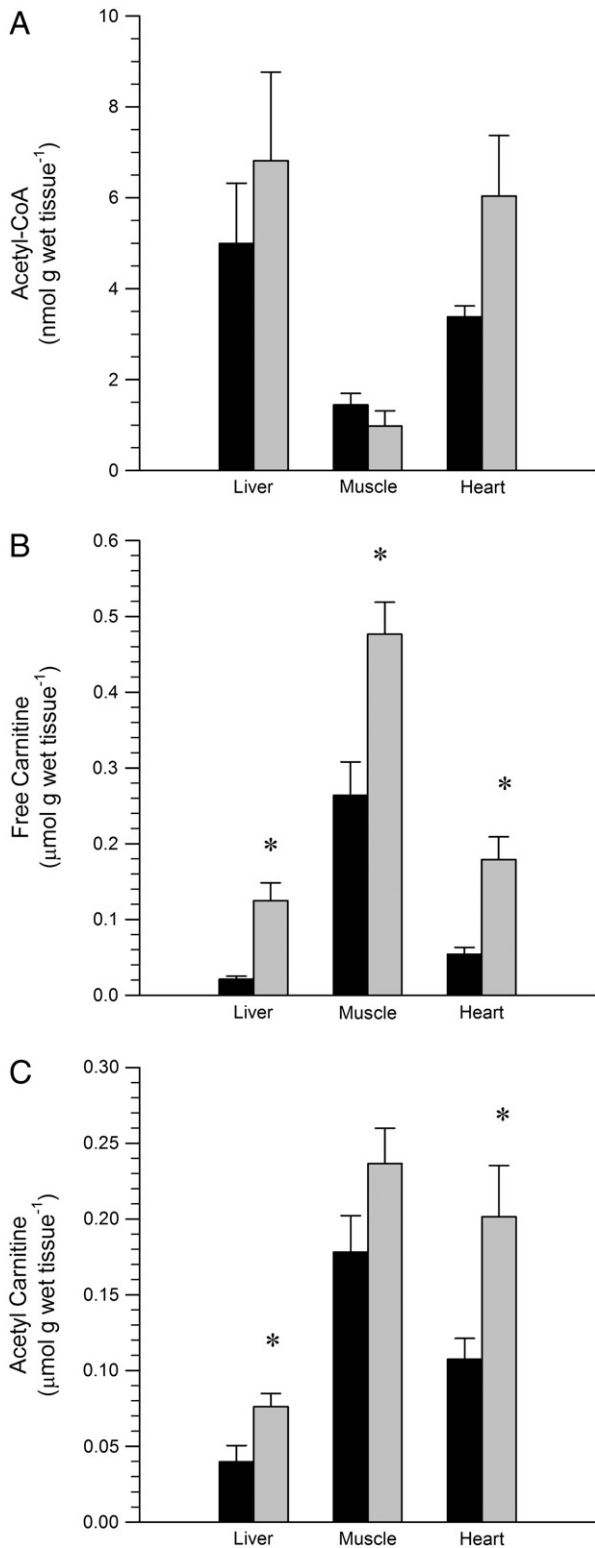
Fig. 5. Liver, muscle and heart acetyl-CoA (A) free carnitine (B) and acetyl-carnitine (C) concentration of active (black bars) and estivating (grey bars) frogs. Values are means ± SEM; n = 6–8. * indicates a significant difference between active and estivating frogs (P < 0.05).

immediately after estivation, but a close relative, *Ceratophrys cornuta,* are known to begin breeding following the first rains of the wet season (Duellman and Lizana, 1994), suggesting that reproduction in this species may follow estivation if it occurs.

Fuel selection during estivation is thought to be characterized by the primary reliance on lipids and protein to fuel metabolism, while carbohydrates have a much reduced contribution (Jones, 1980; Cowan and Storey, 2002; Frick et al., 2008a,b). The reduction in mass, and complete disappearance in some cases, of fat bodies seems to support the heavy reliance on lipids as an oxidizable fuel during estivation (Table 1). Lipids are beneficial due to the ease of storage and the high energy density, when compared to other sources such as carbohydrates and protein (Weber, 2011). The utilization of protein permits gluconeogenesis for tissues that primarily use carbohydrates and for urea production for osmotic balance in the face of dehydration stress (Balinsky, 1981); however, the utilization of protein as an oxidizable fuel is thought in most animals to necessitate the dismantling of cellular and structural components, albeit this notion has not been investigated in anurans. In contrast, carbohydrate stores are maintained, most likely for the exertion required to return to the surface following an estivation event, as suggested by Frick et al. (2008a).

The liver is a multifunctional organ that contributes to ketone body production, carbohydrate and amino acid metabolism, and is reduced in size during estivation (Table 1). The reduction in size could be due to protein catabolism and liberation of amino acids for gluconeogenesis and urea production, as seen in spadefoot toads (Cowan et al., 2000) or due to use of stored lipid and glycogen. Decreases in liver glycogen content was also observed in the *C. ornata* during estivation (Fig. 2B), suggesting that carbohydrates are being used as an energy source, despite glycogen sparing as found in other estivating animals such as the lungfish (Frick et al., 2008a). Other investigators have suggested that estivating animals undergo carbohydrate sparing, as evidenced by the reduction in glycogen phosphorylase and glycolytic enzyme activities (Cowan and Storey, 2002; Frick et al., 2008a) and utilize different substrates in their place. Glycolytic and glycogenolytic enzymes were not analyzed, but pyruvate dehydrogenase, a key regulator in carbohydrate metabolism, was not depressed during estivation in *C. ornata* (Fig. 3A), suggesting that carbohydrates are not spared from oxidation in this species during estivation. It is surprising however that despite large declines in fat storage, there is no evidence of an inhibition of PDH in any tissue examined. PDH regulates pyruvate entry into the citric acid cycle and its activity is heavily influenced (inhibited) by the products of lipid oxidation (Randle, 1995). The lack of an inhibition of PDH in tissues examined during estivation suggests that carbohydrates may in fact be used as an energy source by the frog during estivation, which is further evidenced by the greatly reduced glycogen levels in the liver (Fig. 2B). The increase in CS activity (Fig. 3B) suggests an increase in mitochondrial volume density enhancing aerobic capacity and the increase in free carnitine (Fig. 5B) suggests that the fatty-acyl carnitine esters are reduced, indicating a possible increase in fatty acid oxidation. The exceptionally high levels of acetylcarnitine in the liver (Fig. 5C) further support increased aerobic capacity in the liver and increased oxidation of fatty acids. The formation of ketone bodies in the liver is another possible fate of the accumulated acetyl-groups (acetylcarnitine) and their release into circulation could support aerobic metabolism in tissues incapable of oxidizing fatty acids, particularly nervous tissue (McGarry and Foster, 1980; Laffel, 1999). Unfortunately, analysis of circulating concentrations of ketone bodies could not be performed due to small plasma volumes.

Cardiac function is depressed in spadefoot toads (Seymour, 1973b), salamanders (Gehlbach et al., 1973) and lungfish (Delaney et al., 1974; da Silva et al., 2008) during estivation, due in part to the reduction in metabolic rate and reduced demands for the circulation of oxygen and oxidizable substrates. However, that may not be the case in C. ornata who do not show substantial decreases in metabolic rate in response to

of the gonads is important during estivation. Since some estivating animals often have a short window to reproduce (Seymour, 1973a), *C. ornata* may maintain gonad mass or potentially continues gonadal development during estivation in order to quickly reproduce once estivation breaks. It is unknown if *C. ornata* reproduces

estivation. At the biochemical level, there were no differences in enzyme activities between active and estivating frogs suggesting that overall capacity for ATP production in the heart during estivations does not change. Furthermore, cardiac glycogen levels are not significantly different between estivating and active frogs (Fig. 2B), indicating that intracellular stores of carbohydrates are not used as a fuel source in cardiac tissue. Likewise, skeletal muscle from the leg behaves similarly to cardiac tissue in terms of the enzymatic and metabolite profile. The enzymatic data suggests that both carbohydrates and fatty acids are used, but the accumulation of free carnitine in muscle supports the notion that lipid may be the primary oxidizable fuel. However, estivating frogs are inactive, and muscle-specific metabolic rate suppression does occur in estivating Australian frogs (Kayes et al., 2009).

In order to survive a dry environment, animals must be able to cope with deficiencies in water availability. Some South American, African and Australian frog species have been shown to form a cocoon of shed skin, which acts as a physical barrier to slow the loss of water across the integument into the immediate environment, including the species in this study (McClanahan et al., 1976; Loveridge and Withers, 1981; Cartledge et al., 2006). In addition to the benefits afforded by the formation of a cocoon, modifications to the internal solute profile during estivation also acts to reduce the flux of water across the integument and out of the animal (Withers and Guppy, 1996). The large increase in plasma osmolality suggests that there is an accumulation of solutes within the plasma; however, in the present study a detailed analysis was not possible due to low plasma availability. The increase in osmolality acts to prevent the loss of water to the surrounding environment by decreasing the osmotic gradient between internal fluids and the environment (Withers and Guppy, 1996). It has been shown that amphibians utilize urea as the primary osmolyte to increase the osmolality of their fluids during dehydration stress, through a combination of urea retention and alterations in amino acid metabolism (McClanahan, 1972; Balinsky, 1981).

In some respects, *C. ornata* respond characteristically of what an estivating anuran undergoes during an estivation event, such as accumulation of solutes within internal fluids and behavioral adaptations such as burrowing (Withers, 1995; Withers and Guppy, 1996). Metabolically, however, these estivating frogs do not perform as other well studied estivators, showing evidence of both fatty acid utilization and lacking any indication of metabolic rate suppression or glycogen sparing. This is in contrast to other previously studied estivators (Cowan et al., 2000; Frick et al., 2008a,b), which seem to reduce their reliance upon carbohydrates and instead switch to a more lipid and protein based metabolism in addition to metabolic suppression. These differences may reflect an environmental influence upon arid adaptation and their effect upon inter and intraspecific populations, since this species inhabits a wide array of habitats with different environmental regimes (Canziani and Cannata, 1980).

Acknowledgments

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