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Characterization of circannual patterns of metabolic recovery from activity in *Rana catesbeiana* at 15°C

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Summary

We characterized carbohydrate metabolism following activity in the American bullfrog, *Rana catesbeiana*, and compared whole body metabolic profiles between two seasons. Forty-eight adult male *Rana catesbeiana* were chronically cannulated and injected with [U-^{14}C]L-lactic acid sodium salt in either summer (June) or winter (January) after acclimation for 2 weeks at 15°C with a 12 h:12 h L:D photoperiod. Following injection with [^{14}C]lactate, frogs were either allowed to rest for 240 min (REST), hopped for 2 min on a treadmill and immediately sacrificed (PE), or hopped for 2 min on a treadmill and allowed to recover for 240 min (REC 4). Exercise caused a significant increase in blood lactate level from 2.7±0.1 mmol l^{-1} at rest to 17.0±2.1 mmol l^{-1} immediately following exercise. This increase persisted throughout the recovery period, with average blood lactate level only reduced to 13.7±1.1 mmol l^{-1} after 240 min of recovery, despite complete recovery of intramuscular lactate levels. Lactate levels were not significantly different between seasons in any treatment (REST, PE, REC4), in either gastrocnemius muscle or blood. The vast majority of [^{14}C]lactate was recovered in the muscle, in both winter (86.3%) and summer (87.5%). Season had no effect on total amount of ^{14}C label recovered. [^{14}C]Lactate was measured in the forms of lactate, glucose and glycogen, in the liver and the muscle sampled. The most robust difference found in seasonal metabolism was that both the liver and the gastrocnemius contained significantly higher levels of intracellular free glucose under all treatments in winter. These data suggest that, overall, bullfrogs accumulate and slowly clear lactate in a manner quite similar to findings in fish, other amphibians and lizards. Additionally, our findings indicate that lactate metabolism is not highly influenced by season alone, but that intracellular glucose levels may be sensitive to annual patterns.

Key words: season, lactate, exercise, metabolism, ectotherm, bullfrog, blood pH.

Introduction

It has long been recognized that ectothermic vertebrates such as fish, frogs and lizards rely heavily upon carbohydrate metabolism to fuel activity. Although decades of study have thoroughly characterized the basic pathways of mobilization, production and utilization of glucose, lactate and glycogen, much is still unknown, especially in amphibians, about the regulation of these systems. Even less literature exists examining how these pathways respond to fluctuating external environment in any vertebrate. Previous studies have demonstrated that circannual or seasonal conditions elicit myriad changes in carbohydrate metabolism in ectotherms. It is unclear, however, if changes in metabolic pathways are a response to temperature, photoperiod, or endocrine-mediated endogenous circannual rhythms. In this study, we evaluated basic parameters of post-activity carbohydrate metabolism, and focused specifically on the physiological fate of lactate in a North American amphibian, *Rana catesbeiana*. We then investigate if these parameters respond to changes in season in the absence of changes in temperature or photoperiod.

Studies on carbohydrate metabolism in amphibians have established a basic pattern of accumulation and clearance of lactate following activity for these animals. Following forced activity, under standard laboratory conditions (20–25°C, 12 h:12 h L:D photoperiod), amphibians accumulate lactate rapidly. For example, plasma lactate peaks almost immediately following exercise in *Rana catesbeiana* (Hutchison and Miller, 1978; Putnam, 1979) (for a review, see Bennett, 1982). Lactate production in the muscle follows a similar pattern, but clearance continues long after oxygen consumption has returned to resting values (for a review, see Gleeson, 1991). Muscle lactate utilization in ranids follows a pattern established for other ectotherms, with most intramuscular lactate recycled to glycogen (Fournier and Guderley, 1992). Data from a radioisotope tracer study in *Bufo americanus* indicates that less than 1.5% of labeled lactate injected into an exercising toad is detected as expired CO_2 (Withers et al., 1988), which is

surprising given the relatively high aerobic scope of toads compared to ranid amphibians (Carey, 1979; Putnam, 1979; Putnam and Bennett, 1983). To our knowledge no radioisotope data exist demonstrating the metabolic fate of lactate in a ranid. Overall, the available literature suggests a metabolic recovery strategy in amphibians of both intracellular production and sequestration of lactate, as well as uptake of circulating plasma lactate, mainly by muscle tissue, which is subsequently used to replenish glycogen stores (Putnam, 1979; Withers et al., 1988; Fournier and Guderley, 1992).

With this basic pattern established for carbohydrate metabolism under a single set of conditions, it is important to consider that most amphibians, especially those ranids that inhabit far northern regions of North America, rarely actually exist under such homogenous conditions as those in the laboratory. Species such as the American Bullfrog (*Rana catesbeiana*) that successfully inhabit a range from British Columbia and Nova Scotia south into Mexico (Lannoo, 2005) must acclimatize to significant seasonal climate changes. Activity in the form of migrations, hunting and predator avoidance must occur throughout the year under a variety of ambient conditions. Active swimming by *Rana pipiens* in water temperatures very close to freezing in a Canadian stream has been reported (Cunjack, 1986), and Cunjack suggests that the over-wintering frogs may change microhabitats frequently in order to seek out water with higher oxygen content. Given that the metabolism of ectothermic vertebrates fluctuates with thermal environment, properly allocating, for example, lactate to hepatic rather than intramuscular glycogen stores, is exceedingly important when environmental temperature varies widely. Very little is known about regulation of carbohydrate metabolism in response to changes in season or environmental conditions, and almost nothing is known about lactate metabolism alteration, specifically. In this study, we provide insight into this question, using the bullfrog as a model species that experiences significant seasonal changes in its environment.

It has already been established that several aspects of carbohydrate metabolism do fluctuate seasonally in amphibians. Examples of metabolic parameters that change seasonally in amphibians include metabolite levels (Byrne and White, 1975), oxygen consumption (Bícego-Nahas et al., 2001), and intrinsic skeletal muscle properties (Girgenrath and Marsh, 2003). Circulating levels of (and sensitivity to) epinephrine, glucagon, and insulin also change with the seasons in frogs (Hanke and Neumann, 1972; Farrar and Frye, 1977; Schlaghecke and Blum, 1981). Hepatic glycogen levels in *Rana esculenta* were found to be 6.5× higher in winter frogs than summer frogs, with a correlated increase of activity in the enzymes that control glycogen deposition (Scapin and Di Giuseppe, 1994).

The functional significance of circannual rhythms has been widely speculated on, and may include preemption of freezing conditions (King et al., 1995), protection of glycogen stores for spring emergence from hibernation (Scapin and Di Giuseppe, 1994), and support of glycolytic *versus* oxidative metabolism in potentially hypoxic over-wintering habitats (Tattersall and Boutlier, 1997). It is not known if lactate metabolism is similarly seasonally sensitive. It is also not known if post-exercise glucose homeostasis, gluconeogenesis and glycogenesis respond to time of year.

The purpose of this study was twofold. First, to trace the fate of lactate post-exercise, and create a basic profile of carbohydrate metabolism in the American bullfrog *Rana catesbeiana*, including measurements of extracellular acid–base balance, oxygen consumption, and blood and tissue metabolite profiles. Second, we sought to characterize the metabolic response to activity in the American bullfrog at a seasonally neutral temperature (15°C) in winter (January) and summer (June) months, in order to determine if ‘seasonal’ changes in carbohydrate metabolism are a result of an underlying circannual component, or simply a response to an acute environmental condition, such as temperature or photoperiod. We predict that glycogen stores will be increased in winter months even when the animals are acclimated to a seasonally neutral temperature, and that lactate and glucose metabolism (at rest and in response to activity), will be sensitive to season even in the absence of change in environmental condition.

Materials and methods

Frogs

Adult male *Rana catesbeiana* Shaw 1802 between 85–200 g were purchased from a commercial supplier (Rana Ranch, Twin Falls, ID, USA) that raises frogs with exposure to natural photoperiod. Frogs were received in late May 2005 for summer experiments, and in January 2005 for winter experiments. Animals were acclimated for 2 weeks at 15°C (12:12 photoperiod) with constant access to water and fed 4-week-old crickets 2–3 times per week. Animals were fasted for at least 3 days before experimentation.

Cannulation

Frogs were submerged in neutralized 0.75% tricaine methanesulfonate (MS-222, Sigma, St Louis, MO, USA; cat. no. A5040) solution until fully anaesthetized (approximately 10–20 min). A 2.5–5.0 cm parasagittal incision was made to expose the abdominal cavity directly below the heart, with care taken to avoid (1) severing the mid-ventral vein or (2) damaging the sternal cartilage. A cannula (3-French Soft PU intravascular tubing, bpULD-T30, Instech Solomon, San Antonio, TX, USA) was inserted 5–6 mm into the left systemic arch. The left systemic arch was chosen because it supplies the abdominal viscera rather than more hypoxia-sensitive organs such as the kidney, which are supplied by the right systemic arch (Gilbert, 1965). The cannula was flushed with 0.015% heparin in 0.9% saline, and secured to the vessel using 000 silk surgical sutures. The cannula was run subcutaneously and exited the cavity caudo-dorsally. All surgeries were completed within 2 h. Animals were excluded from experimentation if approximately >1 ml of blood loss occurred during surgery.

Frogs were allowed to recover from surgery for 24 h prior to experimentation based on the results of the following control experiments.

Control experiments

In order to ensure that our cannulation procedure and serial blood sampling were not affecting metabolism, we performed two initial experiments on resting animals. Four adult male *Rana catesbeiana* were chronically cannulated. Blood was sampled *via* the cannula at 0, 6, 24 and 30 h following the surgery and measured for pH using a BMS 3 MK2 analysis system (Radiometer, Copenhagen, Denmark) with a glass capillary electrode. Samples were also analyzed for lactate concentration (Gleeson, 1985). In a separate experiment designed to test the effect of serial sampling on lactate and pH levels, four adult *Rana catesbeiana* were chronically cannulated and allowed to recover for 24 h. Blood was sampled (250 μl) *via* the cannula at rest and then every 60 min for 240 min, totaling six separate 250 μl blood samples (equal or greater than the total volume of blood removed in the following experiments). Whole blood samples were analyzed for pH and plasma samples were analyzed for lactate levels as previously described. Data were statistically compared utilizing a one-way ANOVA, Fisher's PLSD to test for significant ($P\leqslant0.05$) differences between treatment groups. This statistical analysis was used to compare all treatment groups in this study, with the exception of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) data, which were analyzed utilizing unpaired *t*-tests at individual time points.

To ensure the most accurate approximations of organ mass in *Rana catesbeiana*, we dissected six adult males (mass= 178.0±2.8 g) and recorded the mass of the individual organ (blotted free of blood) for use in calculations of the tracer portion of the study.

Experiments

Frogs were allowed to rest in individual containers undisturbed for 240 min on the day of experimentation, with $\dot{V}_{O_2}$ monitored continuously. At the end of the initial rest period, animals were injected with 1 μCi (winter) or 2 μCi (summer) of 50 μCi 0.5 ml^{-1} [U-^{14}C]L-lactic acid sodium salt (MP Biomedicals, Irvine CA, USA). The higher dosage used in summer was to improve the absolute amount of radioactivity captured in respiratory CO_2 samples. All isotopic data are expressed either as percentage of injected dose or are divided by the specific activity of the lactate pool and expressed as μmol lactate converted g^{-1} tissue 4 h^{-1}, thus negating any effect of variable dosage on either data interpretation or the reported data themselves.

Following 10 min of equilibration time post-injection, experimental animals were hopped on a small animal treadmill for 2 min. Control animals were allowed to continue resting with no exercise for 2 min. Blood was sampled *via* the cannula from both groups 12 min after injection (time=0). Depending on treatment group, animals were either sacrificed immediately post-exercise (PE), or allowed to recover for 240 min (REC 4), and then sacrificed. The control group was rested for 240 min (REST) and then sacrificed. Blood was sampled at rest, and every 60 min from time=0 and immediately preserved in six volumes of ice-cold 6.0% $HClO_4$. Whole blood was also rapidly collected from the cannula and analyzed for pH using a radiometer BMS 3 MK2 analysis system with a glass capillary electrode, calibrated to measure pH at 15°C. Respiratory gas exchange was continuously measured throughout recovery using an open flow respirometry system. Downstream air was dried using Drierite desiccant (W. A. Hammond Drierite Company Ltd, Xenia, OH, USA) before entering the analyzer system. Samples were then analyzed by an Anarad, Inc. AR-411 carbon dioxide analyzer and an Applied Electrochemistry S-3A O analyzer. $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ (ml g^{-1} h^{1} STPD; standard temperature and pressure dry) were calculated as per Withers (Withers, 1977) and recorded using LABVIEW data acquisition. All expired CO_2 was then trapped in 24 ml of 1:3 ethanolamine:methylcellusolve, collected and replaced every 30 min, and later analyzed for ^{14}C activity by scintillation counting. At the end of the 240 min experiment, animals were sacrificed via rapid decapitation using a small animal guillotine. Legs, liver and heart (heart in summer only) were quickly dissected out and dropped into liquid nitrogen. The rest of the carcass was blended in four volumes ice cold 6.0% $HClO_4$ and the homogenate saved for later determination of ^{14}C activity. All methods were approved by the University of Colorado Internal Animal Care and Use Committee.

Metabolite determination

Acidified whole blood samples were kept frozen at –70°C for <6 months, thawed and centrifuged (7200 ***g*** for 10 min). The supernatant was analyzed for lactate (Gleeson, 1985) and glucose concentrations (Bergmeyer and Bernt, 1974). Tissues were kept frozen at –70°C for <6 months, and then homogenized in six volumes ice cold 6.0% $HClO_4$. Homogenates were then centrifuged at 7200 ***g*** for 10 min, and the supernatant analyzed for lactate and glucose as above. Metabolite values for all the tissues were corrected for extracellular spaces of 10%, 26% and 30%, for muscle (Ling and Kromash, 1967), liver (Devireddy et al., 1999) and heart (Armstrong et al., 1969), respectively. Glycogen content of tissues was analyzed by ethanol precipitation of glycogen, subsequent breakdown with amyglucosidase (Keppler and Decker, 1974) followed by determination of glucose levels as referenced above.

^{14}C activity

Supernatant from blood and tissue samples, as well as trapped CO_2 in ethanolamine and methycellusolve solution, were analyzed for activity of injected ^{14}C. Aliquots (100 μl) of blood and tissue samples were separated into lactate and glucose using a Dowex ion exchange column as described in Donovan and Gleeson (Donovan and Gleeson, 2006). These samples, as well as aliquots of the precipitated glycogen were then analyzed using scintillation counting for ^{14}C activity. Samples (1 ml) of trapped CO_2 were mixed with 3 ml of

Scintiverse Scintillation cocktail (Fisher Scientific, Houston, TX, USA) and aqueous samples (plasma, tissue) were mixed with 3.5 ml of Scintisafe Plus 50% Scintillation cocktail (Fisher Scientific, Houston, TX, USA). Samples were analyzed using a Wallac Model 1204 scintillation counter with internal quench correction. For ease of comparison, all data presented and discussed are expressed in terms of percentage of counts returned.

Results

Initial controls and tissue mass

Cannulation resulted in an immediate lactacidosis. Both pH (Fig. 1A) and lactate (Fig. 1C) returned to resting levels within 6 h and remained at these levels for 30 h of sampling following surgery. Serial sampling had no significant effect on either blood pH (Fig. 1B, P>0.05) or lactate (Fig. 1D, P>0.05). Results of our tissue mass analysis are reported in Table 1 and indicate that muscle constitutes 41% of a frog's mass.

Blood pH, oxygen consumption, and metabolites – summer

Following activity in the 'summer' frogs, both $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ immediately increased three to four times but steadily returned to resting levels within 70 min. Peak $\dot{V}_{O_2}$ following activity reached 0.12 ml O_2 g^{-1} h^{-1} and peak $\dot{V}_{CO_2}$ was 0.33 ml CO_2 g^{-1} h^{-1} (Fig. 2). Blood pH dropped rapidly following exercise (Fig. 3A) and was inversely proportional to plasma accumulation of lactate (Fig. 3B). Blood pH continually recovered from the initial acidosis until reaching resting levels within 240 min, rising on average 0.1 pH units h^{-1} (Fig. 3A). Within the same time period, blood lactate remained elevated 2–3 times above resting levels, with clearance in the 240 min of recovery equal to about 30% of lactate produced by activity (Fig. 3B). Resting blood glucose levels remained relatively stable over the 4 h experiment, and did not differ significantly at any time point from the average blood concentration of 0.72±0.1 mmol l^{-1} (P>0.05). Blood glucose concentration progressively increased following exercise and peaked at 1.6 mmol l^{-1} at 180 min of recovery, and was significantly elevated from resting values at all sampling intervals following exercise (Fig. 3C).

Table 1. *Organ mass in Rana catesbeiana*

Organ	% of whole animal (by mass)
Heart	0.5±0.02
Liver	3.0±0.3
Viscera	12.6±1.3
Skin	12.6±0.4
Skeletal muscle	40.9±1.9
Bones	7.9±0.3
Head	9.0±0.3
Other	13.5±1.9

Values are means ± s.e.m.; N=5.
Mean body mass = 178 g.

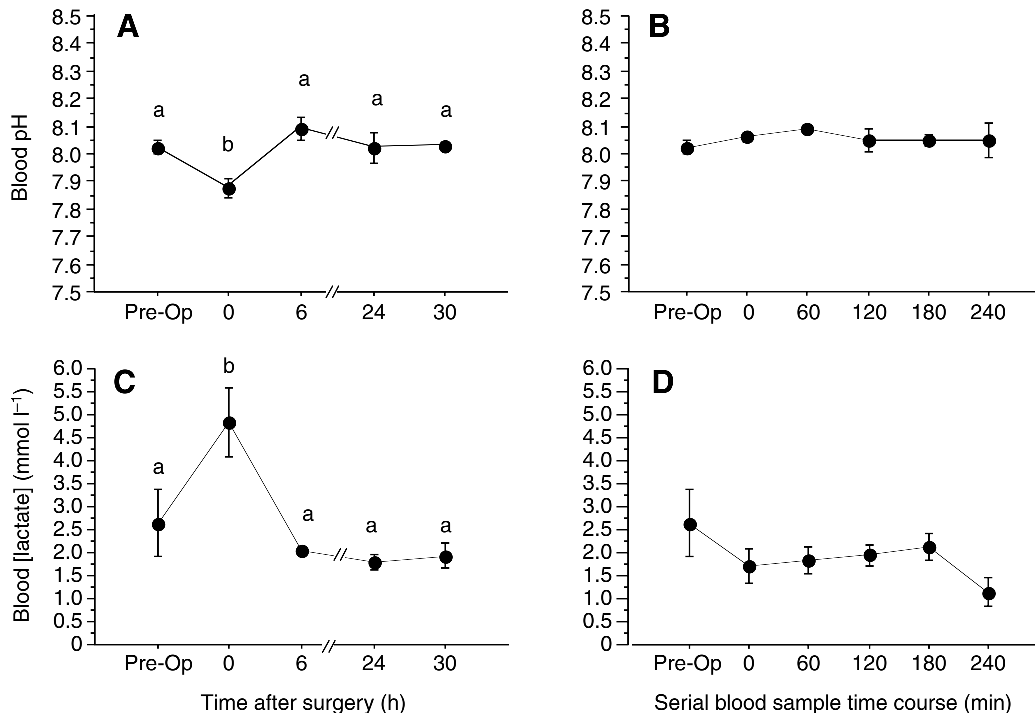

Fig. 1. The effect of cannulation surgery and repeated blood sampling on plasma lactate and blood pH in *Rana catesbeiana*. (A) Blood pH and (C) plasma lactate levels following a typical (>2 h) cannulation surgery. Differing letters indicate significant differences between treatments (P<0.05, N=4). (B,D) The effect of repeated blood sampling on blood pH (B) and plasma lactate (D; P>0.05, N=4). Values are means ± s.e.m.

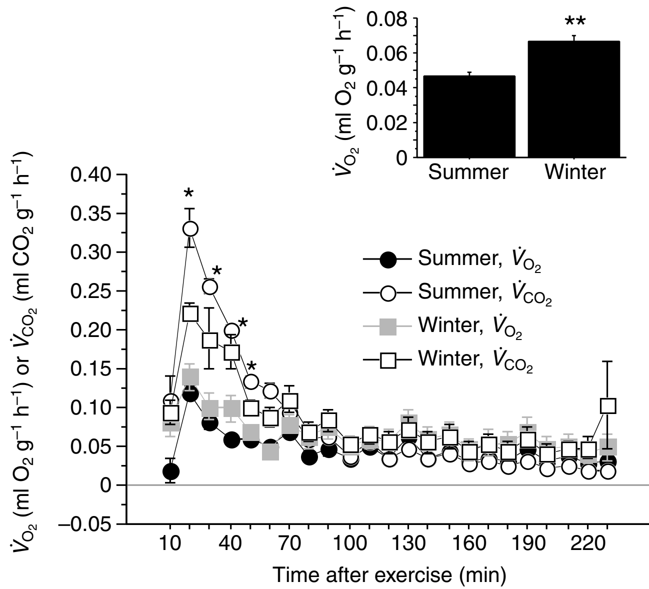

Fig. 2. The effect of exercise performed in June and January in *Rana catesbeiana* on the rate of oxygen consumed ($\dot{V}_{O_2}$; ml O_2 g^{-1} h^{-1}) in summer (black circles) and winter (grey squares) and the rate of carbon dioxide produced ($\dot{V}_{CO_2}$; ml CO_2 g^{-1} h^{-1}) in summer (open circles) and in winter (open squares). Values are means ± s.e.m. Asterisks indicate times at which both $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ are significantly different from resting frog (REST) values (P>0.05, unpaired t-test by time, N=6–8). Inset shows 240 min average of $\dot{V}_{O_2}$ in winter and summer post-activity; **significant difference (P<0.05, N=6–8) between identical treatments and gases measured in different seasons.

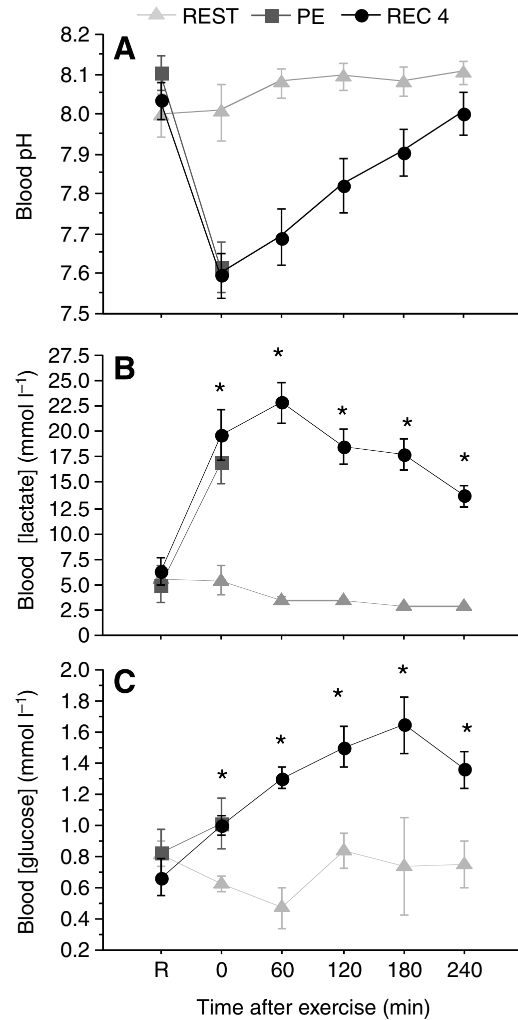

Fig. 3. The effect of exercise on blood (A) pH, (B) lactate and (C) glucose concentrations in *Rana catesbeiana* at 15°C in June. Closed triangles, resting frogs (REST); closed circles, exercise + 4 h recovery (REC 4); closed squares, immediately post-exercise (PE). Values are means ± s.e.m. *Significant difference from REST values (P<0.05, N=6–8).

Tissue metabolites

Intracellular lactate concentrations in the gastrocnemius muscle, the liver and the heart, increased significantly following activity as would be expected (P<0.05, Fig. 4A). Following exercise, lactate levels in the gastrocnemius muscle increased from resting levels of 9.2±3.5 mmol l^{-1} to an average of nearly 40.0 mmol l^{-1}. Muscle lactate levels were statistically indistinguishable from resting levels by 240 min of recovery (P>0.05). This is in contrast to the pattern of incomplete lactate clearance during recovery period measured in both heart and liver tissues (Fig. 4A). Liver cells were found to have an average resting lactate concentration of 4.2±1.3 mmol l^{-1}. Immediately following 2 min of hopping, liver lactate levels doubled, and remained significantly elevated throughout the duration of recovery. Heart tissue followed a similar pattern, with low resting lactate concentrations (2.0±0.1 mmol l^{-1}) that rose immediately following activity to approximately 10 mmol l^{-1}. Heart lactate levels remained significantly elevated from resting values over the 240 min of recovery (P<0.05, Fig. 4A).

Exercise had no significant effect on liver glycogen stores which remained between 200–300 mmol glycosyl units l^{-1} for the duration of the experiment in both resting and exercised animals (P>0.05, Fig. 4C). Muscle glycogen, on the other hand, fell significantly from 28.7±7.7 mmol glycosyl units l^{-1} at rest to 15.8±1.2 mmol glycosyl units l^{-1} after exercise (Fig. 4D). In spite of the apparent complete clearance of intracellular lactate from the gastrocnemius by 240 min of recovery, muscle glycogen levels remained significantly depleted compared to resting values by the end of the same time course (P<0.05, Fig. 4D). Season had no significant effect on either liver or muscle glycogen levels (P>0.05, Fig. 4C,D).

Fate of [^{14}C]L-lactate

Tissue and gas analyses accounted for 50–70% of the injected [^{14}C]lactate. Of this fraction, approximately 60–70% of the [^{14}C]lactate was converted to another form during the 240 min of recovery (Fig. 5, Table 2 and Appendix). The measured pattern of lactate dispersal was the same regardless of whether the frog rested for 240 min or was exercised and allowed to recover for 240 min (P>0.05, Fig. 5). Of that

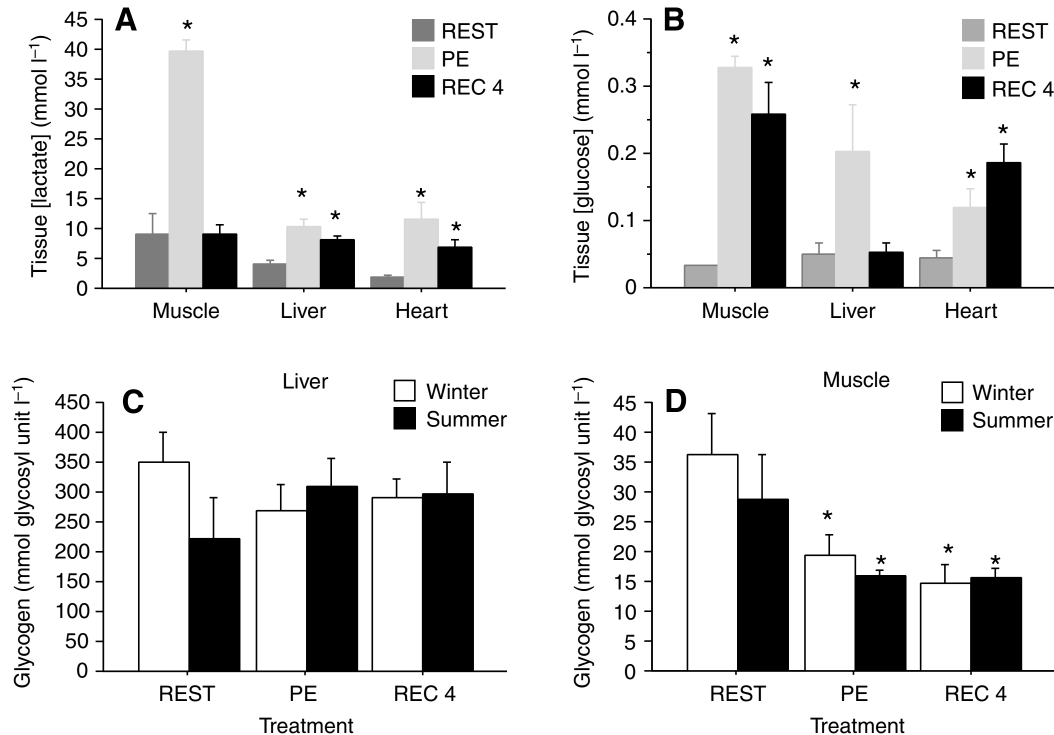

Fig. 4. (A,B) Tissue lactate (A) and glucose (B) concentrations at rest (REST, dark gray bars), immediately post-exercise (PE, light gray bars), and after 4 h of recovery (REC 4, black bars). Values are means ± s.e.m. *Significant difference from REST ($P<0.05$, N=6–8). (C,D) Tissue glycogen stores (± s.e.m.) in liver (C) and muscle (D) in *Rana catesbeiana* in January (white bars) and June (black bars) at rest (REST), immediately post-exercise (PE), and after 4 h of recovery (REC 4).

60%, the majority (40–45%) of lactate cleared was gluoconeogenically converted to glycogen stores in the muscle and the liver, whereas another 10% was gluconeogenically converted to glucose in tissue and blood glucose pools. Approximately 10% of the lactate was oxidized (Fig. 5). Five- to tenfold as much *de novo* glucose and glycogen was deposited in skeletal muscle (per gram) as was deposited in liver (Fig. 6).

Seasonal changes in metabolic profile

Most parameters measured did not change significantly based on season alone when temperature and photoperiod were held constant (Table 2).

There was more lactate carbon incorporated into liver glycogen than liver glucose, although this trend was only significant in the summer (Fig. 6, Appendix). The gastrocnemius muscle converted the majority of its lactate into glycogen in the winter ($P<0.05$), but equally into both glucose and glycogen in the summer. The muscle appeared to demonstrate an overall increased gluconeogenic and glyconeogenic function in summer, converting up to two to three times more lactate to glycogen and glucose than in winter, although this trend was not significant (Fig. 6). Lactate deposition in liver was not influenced by season. Activity did not significantly increase either liver or muscle conversion of lactate to glucose or glycogen in either season (Fig. 6, Table 2, Appendix).

Tissue glucose

Of all parameters examined, levels of free intracellular glucose demonstrated the most significant fluctuations based solely on season. Although all three tissues (liver, heart, muscle) demonstrated an identical response to activity (immediate increases in glucose that were not reduced to resting values within 4 h of activity, $P<0.05$, Fig. 7), tissues from January experiments contained two to four times as much glucose at all sampling periods as those collected in June (Table 2, Fig. 7). Muscle and heart glucose levels remained significantly elevated for the duration of the 4 h recovery, while liver samples returned to resting levels in both seasons.

$\dot{V}_{O_2}$ *and* $\dot{V}_{CO_2}$

The average $\dot{V}_{O_2}$ of frogs in winter over 240 min was significantly higher than that of frogs in summer following exercise ($P<0.05$; Fig. 2, Table 2), although no significant difference was found between seasons at individual 30 min intervals (Fig. 2). Resting $\dot{V}_{O_2}$ was not different between seasons ($P>0.05$; Fig. 2, Table 2). The average $\dot{V}_{CO_2}$ of winter frogs was not different from summer frogs ($P>0.05$) when averaged over the 4 h of recovery. However, the peak $\dot{V}_{CO_2}$, as well as the $\dot{V}_{CO_2}$ averages at 20, 30 and 40 min of recovery, were found to be significantly higher in summer frogs ($P<0.05$; Fig. 2).

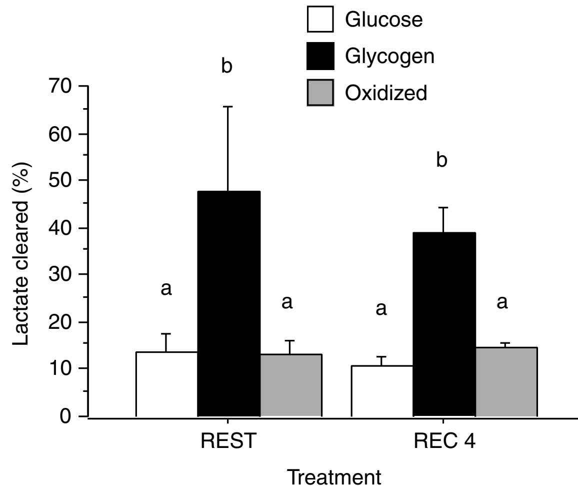

Fig. 5. Percentage (mean ± s.e.m.) of injected labeled lactate that was converted throughout the body to glucose (white bars) or glycogen (black bars) or oxidized (grey bars) over 4 h in either resting (REST) or exercised frogs recovered for 4 h (REC 4) in June. Different letters indicate significantly different metabolic fate of lactate between groups ($P<0.05$, $N=7–8$).

Discussion

Characterization of metabolic recovery from activity: summer

Lactate production and recycling in bullfrogs were found to follow a pattern typical for other exercised ectotherm, and were not altered between seasons. The general pattern of blood lactate accumulation and metabolism following activity in *Rana catesbeiana* at 15°C (Fig. 3B) is in keeping with data reported previously for amphibians (Fournier and Guderley, 1992), lizards (Gleeson and Dalessio, 1989) and fish (Milligan and Girard, 1993). The increase in both tissue and blood lactate levels are typical of an animal performing power sprint type exercise, supported mainly by glycolytic white muscle (Putnam and Bennett, 1983).

The intra–extracellular concentration gradient for lactate over the course of recovery is not typical for an ectotherms. Fig. 3B clearly shows a lactate clearance pattern in which blood lactate levels remain significantly elevated for the entire course of recovery, despite the complete clearance of excess lactate from the skeletal muscle tissue (Fig. 4A). In contrast to these findings, blood and tissue lactate levels reported by Fournier et al. (Fournier et al., 1994) in *Rana pipiens* demonstrate what could be considered to be a pattern of lactate clearance typically found in reptiles and mammals, in which extracellular levels remain equal to or lower than intracellular levels for the duration of recovery (for reviews, see Gleeson, 1991; Gleeson, 1996). Similar to our findings in bullfrogs, a lactate ‘reversed-gradient’ following exercise recovery was observed in *Rana temporaria*, but only at hibernating temperatures (>7°C) in frogs submerged for several months (Tattersall and Boutilier, 1999). Although the bullfrogs in our study were acclimated to a temperature of 15°C, it is unlikely that this temperature was cold enough to elicit a hibernation response. However, when we repeated this experiment at 25°C the concentration gradient was abolished (Petersen et al., 2006), indicating that temperature may play a role in creating this gradient.

Although monocarboxylate transporter (MCT) isoforms have not been identified in amphibian skeletal muscle, data from Mason et al. (Mason et al., 1986) suggest that lactate export from frog sartorius muscle is driven by lactate/proton co-transport. Evidence for MCT 1 and MCT 4 transporter proteins has been established in fish (Wang et al., 1997; Laberee and Milligan, 1999) and in lizards (Donovan and Gleeson, 2001). Assuming that amphibian skeletal muscle also relies on a lactate/H^+ co-transport system, low temperature may be affecting lactate uptake by reducing transporter protein activity in the muscle and other tissues. Although changes in extracellular pH associated with the large lactate load in the blood could also alter transporter kinetics, blood pH recovers to resting value within the 240 min of recovery while extracellular lactate remains elevated (Fig. 3A,B). Therefore, it is unlikely that alterations in the intra–extracellular pH gradient are driving the exclusion of lactate from the cells. Amphibians may employ an active lactate transporter system in order to maintain this reversed-concentration gradient (Donohoe and Boutilier, 1998), but to our knowledge, no additional evidence has been reported to support this hypothesis. Limited perfusion may also limit the muscle’s ability to take up lactate or glucose. Amphibian studies have clearly demonstrated that the combination of cold submergence (5°C) and hypoxia elicit shunting of blood flow away from vessels feeding muscle beds (Boutilier et al., 1986; Pinder et al., 1992). White muscle tissue specifically is under-perfused at low temperatures in rainbow trout (Barron et al., 1987). More studies are necessary in order to determine if the muscle is limited in protein-facilitated metabolite uptake, or if reduced perfusion is responsible for the gradient observed in this study.

Plasma and muscle glucose increased significantly and remained elevated throughout the course of recovery (Fig. 3C and Fig. 4B). The hyperglycemic plasma response to activity is indicative of the exercise-induced catecholamine activation reported in *Rana pipiens* (Fournier et al., 1994). Yet this is an unusual pattern for ectotherms. In studies on lizards sprinted for 15 s (Donovan and Gleeson, 2006) or sprinted for 5 min (Gleeson and Dalessio, 1989), blood glucose levels remain unchanged. Post-exercise increases in blood glucose are reported to be non-existent (Pagnotta and Milligan, 1991), or minimal (Wang et al., 1994) in rainbow trout. Frogs on the other hand, appear to follow a more ‘mammalian’ pattern, of significant increases in blood glucose in response to activity (Fig. 4B), coinciding with and presumably due to catecholamine release (Fournier et al., 1994). Mammals restore normoglycemia within 1 h of epinephrine clearance (Sigal et al., 1994), whereas significant hyperglycemia was persistent over the 4 h of recovery in this study (Fig. 3C) and that of Fournier et al. (Fournier et al., 1994).

It is unknown whether extended, elevated glucose post-activity confers any functional benefit to recovery in frogs, or may simply be a consequence of low metabolism. However,

Table 2. *Seasonal changes in the metabolic profile of Rana catesbeiana at 15°C*

Parameter	Tissue	Season	Treatment		
			REST	PE	REC 4
pH	Blood	Summer	7.99±0.05	7.62±0.06	8.00±0.05
		Winter	8.10±0.02	7.66±0.07	7.95±0.02
[Lactate] (mmol l^{-1})	Blood	Summer	2.72±0.12	17.01±2.13	13.69±1.10
		Winter	4.76±2.50	20.51±4.35	15.82±1.18
	Muscle	Summer	9.17±3.45	39.69±1.95	9.71±1.78
		Winter	3.67±0.69	29.97±2.43	7.70±1.00
	Liver	Summer†	4.22±1.60	**10.24±1.29**	8.41±2.31
		Winter	3.44±0.98	3.00±1.05	5.36±1.63
[Glucose] (mmol l^{-1})	Blood	Summer	0.75±0.41	1.01±0.45	1.36±0.34
		Winter	0.75±0.29	0.89±0.31	1.11±0.42
	Muscle	Summer	0.18±0.04	1.82±0.10	1.43±0.26
		Winter†	**1.35±0.16**	**2.91±0.26**	**2.51±0.28**
	Liver	Summer	0.27±0.10	1.12±0.39	0.30±0.08
		Winter†	**1.16±0.07**	2.11±0.28	**1.14±0.07**
$\dot{V}_{O_2}$ (ml g^{-1} h^{-1})		Summer	0.04±0.00	N/A	0.06±0.00
		Winter†	0.04±0.00	N/A	**0.08±0.00**
$\dot{V}_{CO_2}$ (ml g^{-1} h^{-1})		Summer†	0.04±0.00	N/A	**0.15±0.00**
		Winter	0.04±0.00	N/A	0.12±0.00
[^{14}C]lactate					
d.p.m. recovered from tissue/breath*	Muscle	Summer	87.5%	N/A	75.8%
		Winter	86.3%	N/A	61.5%
	Liver	Summer	3.5%	N/A	4.7%
		Winter	1.8%	N/A	9.8%
	Oxidised	Summer	9.0%	N/A	19.5%
		Winter	11.9%	N/A	28.7%

Values are means ± s.e.m.

*% of total d.p.m. recovered.

†The average (in bold) is significantly greater than the other season ($P<0.05$, one-way ANOVA, Fisher's PLSD, N=5–8).

the very presence of elevated extracellular glucose levels is intriguing, because it suggests that hepatic and renal tissues may be more involved in blood glucose homeostasis and exercise fuel provisioning than previously suggested (Fournier and Guderley, 1992; Fournier and Guderley, 1993; Fournier et al., 1994; Pagnotta and Milligan, 1994).

Previous research on *Rana pipiens* demonstrated negligible lactate uptake or glucose efflux by the liver (Fournier and Guderley, 1992; Fournier and Guderley, 1993). In fact, after these authors removed the liver from the frog, post-exercise lactate removal rates and blood glucose levels were not significantly different from those of intact animals (Fournier and Guderley, 1993). The increases in circulating glucose (Fig. 4B) might suggest that the liver or possibly the kidneys are breaking down glycogen or gluconeogenically converting triglycerides or protein to glucose. In humans, the kidneys have been shown to be responsible for up to 40% of systemic glucose appearance in response to epinephrine (Stumvoll et al., 1995), a finding that could reconcile the conundrum of normal plasma glucose levels in hepatectomized frogs. Liver is the only tissue examined that returned to resting glucose values by the end of the trial (Fig. 4B), further suggesting possible export of glucose from this tissue. Concomitant elevated hepatic lactate levels are additionally indicative of Cori-cycling. 240 min after cessation of exercise, free glucose in the gastrocnemius muscle remained elevated, and the heart was higher in glucose than immediately following exercise (Fig. 4B), suggesting continued uptake of extracellular glucose.

However, hepatic glycogen reserves were not measurably changed in response to exercise (Fig. 4C), arguing against this interpretation. An intriguing pathway, that frogs are exporting glucose from muscle utilizing glucosidic pathways, has been proposed (Fournier and Guderley, 1993). This hypothesis has not yet been thoroughly tested. The liver of some frogs, albeit in response to freezing temperatures, is nonetheless extraordinarily capable of exporting glucose (Storey, 1987). Our findings of [^{14}C]glucose in the blood suggest that the liver is involved *in vivo* in post-activity glucose production. The role of the liver as a glucose source in frogs remains enigmatic.

Limitations of tracer methodology

Tissue lactate exchange with the pyruvate pool can theoretically limit the interpretation of the isotopic data (Stanley and Lehman, 1988). This exchange may be more problematic in the resting state than during recovery, when the net flux through LDH strongly favors net reduction in the lactate pool size (Gleeson and Delassio, 1989). Under resting conditions, lactate and pyruvate pools are probably closer to

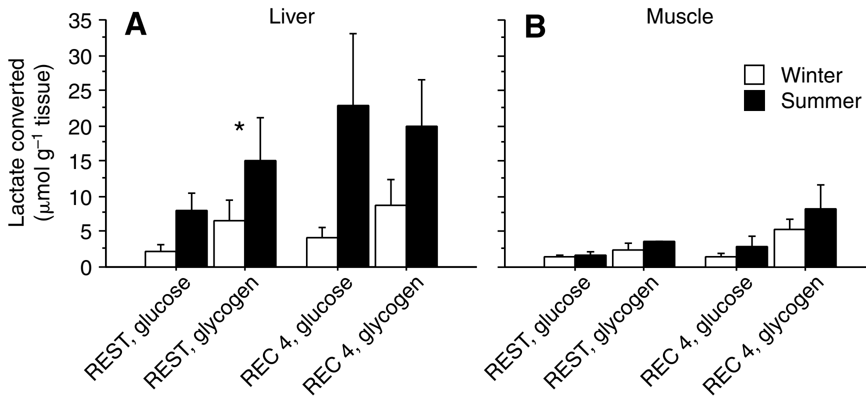

Fig. 6. Lactate conversion (mean ± s.e.m.) to glucose or glycogen in the liver and muscle over 4 h of resting (REST) or 4 h of recovery after activity (REC 4) in January (white bars) or June (black bars). *Significant difference between seasons (*P*<0.05, *N*=7–8).

equilibrium. As a result, label incorporation into glucose, glycogen, or CO_2 does not reflect net lactate removal as much as it reflects lactate pool turnover. Given these limitations we have interpreted resting animal data with caution.

$\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and pH: summer

Both $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ increased immediately following activity, and returned to resting levels within 100 min of recovery (Fig. 2). Withers et al. (Withers et al., 1988) found that toads exercised for 10 min also demonstrated a rapid return to resting oxygen consumption within 1 h, in spite of the persistence of blood lactate elevation. In our study, $\dot{V}_{CO_2}$ returned to resting levels approximately 80 min before respiratory acidosis was significantly alleviated (Fig. 2). This pattern is indicative of an acid–base buffering system that depends on the buffering capacity of bicarbonate (for a review, see Reeves, 1977), and consistent with more recent explanations of buffering from mineralized tissues such as the endolymphatic sac (Warren and Jackson, 2005), and renal and cutaneous proton excretion (for a review, see Boutilier et al., 1992).

The post-activity fate of lactate: summer

The majority of the [^{14}C]lactate metabolized was converted to glycogen or glucose following exercise or rest. Unexpectedly, exercise had no effect on the metabolic fate of lactate (Fig. 5). We might have expected an increase in oxidation of lactate concomitant to increased overall aerobic metabolism following exercise. Our findings in terms of the fate of lactate are in keeping with those reported in toads (Withers et al., 1988) and lizards (Gleeson and Delassio, 1989) that the majority of lactate produced following activity is stored as glycogen, with <20% of lactate being oxidized. This ‘carbon-recycling’ pattern post-exercise is now well established for most ectothermic vertebrates (for reviews, see Gleeson, 1991; Gleeson, 1996). Based on label incorporation, both muscle and liver demonstrated glyconeogenic and gluconeogenic capacity (Fig. 6). The liver, as previously reported in lizards (Gleeson and Delassio, 1989), fish (Milligan and Gerard, 1993) and frogs (Fournier and Guderley, 1992) was found to be of lesser importance to whole body activity recovery than the skeletal muscle. Our data support this (Fig. 6), as the per gram tissue glyconeogenic and gluconeogenic rate of label incorporation in the muscle tissue exceeded that of the liver by 5–10 times; however, the liver does appear to contribute to lactate recycling to some extent.

Seasonal changes in carbohydrate metabolism

Bullfrogs demonstrated surprisingly little change in metabolic profile between seasons when acclimated to a single temperature. Glycogen levels (Fig. 4C,D) were not different between summer and winter after only a 2-week acclimatization period. These data are in contrast to the

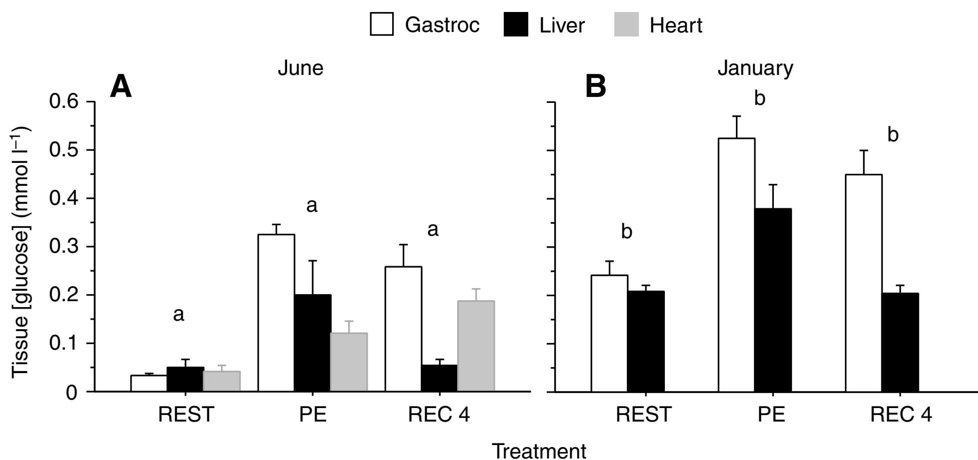

Fig. 7. The effect of season on intracellular glucose concentration in different tissues (gastrocnemius muscle, liver and heart) after acclimation and exercise at 15°C. January tissues had a higher glucose concentration at rest (REST), immediately following exercise (PE), and 4 h after exercise (REC 4). Values are means ± s.e.m. Differing letters denote significant difference between glucose concentrations under identical treatment conditions when the experiment was performed in different months (*P*<0.05, *N*=6–8).

findings of Scapin and Di Giuseppe (Scapin and Di Giuseppe, 1994) who demonstrated a sixfold increase in hepatic glycogen levels in *Rana escualenta* housed in an outdoor terrarium over the course of the year. This is an impressive glycogen loading ability considering that the maximum glycogen ‘supercompensation’ thought possible in rat tissue is twice resting values, and this is only achieved *via* extreme training regimens and creatine phosphate supplementation (Nelson et al., 2001) or treatment with anabolic steroids (Cunha et al., 2005). Our findings in *Rana catesbeiana* suggest that previously reported extreme seasonal glycogen production and storage must be correlated to acute temperature rather than a chronic change between seasons.

No significant difference was found to exist between summer and winter frogs in terms of resting or post-exercise lactate and pH levels. Likewise, circulating glucose levels were not impacted by season alone. However, both liver and muscle glucose levels were significantly higher in January animals at rest and following exercise (Fig. 7). This is an interesting finding in the light of the large body of literature describing the use of glucose by over-wintering frogs as a cryoprotectant (Storey and Storey, 1984; Costanzo et al., 1993; Steiner et al., 2000). However, the mechanism by which glucose accumulates in tissues has clearly been established in some ranids to be triggered not by winter, or even cold temperature, but only by temperatures below zero (Storey and Storey, 1984; Costanzo et al., 1993). Therefore, it is unlikely that the 15°C exposure reported here is eliciting a cryoprotective response. However, older literature has reported that such glucoregulatory hormones as epinephrine (Farrar and Frye, 1977) and insulin are more effective in amphibians in the fall (Hanke and Neumman, 1972) and circulate in higher concentrations in winter (Schlaghecke and Blum, 1981). It is therefore plausible that heightened sensitivity to and/or concentrations of insulin could be responsible for the increased tissue glucose levels found in winter animals (Fig. 7).

The isotope incorporation data suggest that gluconeogenic and glyconeogenic function of the gastrocnemius muscle are increased in summer (Fig. 6). It would make sense that in summer months, when breeding and feeding occur, to have increased gluconeogenic capacity to support substrate homeostasis. This increased conversion of labeled lactate to glucose and glycogen in the muscle does not translate to higher intracellular glucose or glycogen levels at this time of year, even in response to activity. Therefore, this increased capacity may be representative of an overall fuel substrate change. Muscle may rely predominantly on breakdown of stored fat bodies in winter, but switch to carbohydrate sources in the summer. Our data do, in fact, suggest a higher respiratory exchange ratio (RER) value in summer frogs post-exercise (Fig. 2), possibly indicating that more carbohydrate is being metabolized in this season. Donohoe and Boutilier (Donohoe and Boutilier, 1998) report that wild caught frogs in January maintain resting respiratory quotient (RQ), reflective of lipid metabolism, at least for the first 45 days of submergence in the laboratory. The seasonal differences reported here in $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ could be due to seasonal changes in bicarbonate buffering capacity. Another possibility besides changes in bicarbonate buffering capacity is that mineralized tissue buffering capacity is altered between seasons. Warren and Jackson (Warren and Jackson, 2005) have recently demonstrated that *Rana pipiens* utilized both bone and the endolymphatic sacs as sinks for increased proton load following exercise. Changes in the capacity of this system could certainly lead to alteration in levels of CO_2 expired. Future studies should address questions of whole body buffering capacity by season.

Appendix

Seasonal changes in the isotopic metabolic profile of Rana catesbeiana at 15°C

				Treatment	
[^{14}C]Lactate	Tissue	Season	Carbohydrate	REST	REC 4
Specific activity*	Muscle	Summer	Lactate	674±111	424±127
		Winter	Lactate	2295±1611	529±149
	Liver	Summer	Lactate	1454±829	434±43
		Winter	Lactate	488±84	3288±1903
d.p.m. g^{-1} tissue	Muscle	Summer	Glucose	2108±712	1690±252
			Glycogen	8042±2626	4670±1100
		Winter	Glucose	2345±1106	1493±394
			Glycogen	8015±1827	4570±1113
	Liver	Summer	Glucose	869±256	604±165
			Glycogen	1722±774	1949±482
		Winter	Glucose	390±89	406±41
			Glycogen	2001±430	4901±3053

Values are means ± s.e.m. (N=6–8).
*d.p.m. μmol^{-1} lactate.

In conclusion, *Rana catesbeiana* appear to recycle lactate accumulated during exercise into glycogen stores in a manner similar to other amphibians, fish and lizards. Our data suggest an interesting reversed concentration gradient of extracellular to intracellular lactate post-exercise at 15°C that could be due to changes in transporter kinetics. In terms of seasonal changes in carbohydrate metabolism, it appears that intracellular glucose level is the only parameter of those examined that is altered by season alone. Lactate metabolism dose not appear to be altered seasonally. Based on this study, we conclude that previously reported 'seasonal' changes in metabolic profiles in ranid amphibians are likely due to an acute environmental condition rather than a chronic seasonal alteration of metabolism.

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