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# Skeletal muscle substrate utilization is altered by acute and acclimatory temperature in the American bullfrog (*Lithobates catesbeiana*)

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## SUMMARY

**We investigated the effect of acute and acclimatory temperature on the relative contribution of glucose and lactate to metabolism in resting sartorius muscle of the American bullfrog (*Lithobates catesbeiana*). We examined the fate of these metabolites *in vitro* by supplying radiolabeled [$^{14}$C]glucose, [$^{14}$C]lactate and [$^{14}$C]palmitate to isolated muscle bundles from frogs (1) acutely exposed to incubation conditions of 5, 15 or 25°C, (2) acclimated for 2–6 weeks to 5 or 25°C or (3) acclimated for 2–6 weeks to 5 or 25°C and the muscles incubated at 15°C. Under all three temperature conditions tested, net rate of lactate metabolism exceeded that of glucose. Acute exposure to 5°C reduced net rate of glucose metabolism by 15× and net lactate metabolism by 10× as compared with 25°C-exposed tissues. Acclimation to 5°C favored glucose storage as glycogen and increased the proportion of lactate oxidized (*versus* stored or converted to glucose) when compared with 25°C-acclimated tissues. Net rates of storage of lactate as glycogen (glyconeogenesis) were significantly higher in muscles from 5°C-acclimated frogs during incubation at a common temperature of 15°C. These data suggest that lactate is the predominant fuel for resting skeletal muscle over this temperature range, and particularly so under cold conditions. Ready use of lactate as a substrate, and enhancement of glyconeogenic pathways in response to cold acclimation, could play a role in the tolerance of this species to seasonal temperature changes by promoting sequestration and storage of available substrate under cold conditions.**

Key words: lactate, glucose, glycogen, *Rana catesbeiana*, ectotherm, muscle metabolism.

## INTRODUCTION

Substrate preference for cellular metabolism varies between species and individual tissues of vertebrates. Muscle fibers of amphibians are known to predominantly utilize anaerobic pathways for energy at rest and subsequent activity (Bennett and Licht, 1974; Hutchinson and Miller, 1978). Studies have demonstrated clearly that mammalian, amphibian, and reptilian muscles have the capacity to use glucose and lactate as substrate for ATP generation *via* oxidative phosphorylation and for intramuscular glycogen synthesis (Bendall and Taylor, 1970; Connett, 1979; Gleeson, 1985; Bonen et al., 1990). In fact, one of the earliest studies on tissue lactate utilization was performed in amphibian skeletal muscle (Meyerhoff, 1920).

Several substrates are metabolized by vertebrate skeletal muscle, including carbohydrates (glucose), carboxylic acids (lactate) and fats (fatty acids). The major metabolic fates for these substrates in vertebrate muscle tissue are oxidation to $CO_2$, incorporation into glycogen, or carbon exchange between the glucose and lactate pools. Numerous physiological, seasonal and ecological factors undoubtedly play a role in substrate and metabolic pathway selection by skeletal muscle cells of different animals. However, a comprehensive, comparative analysis of cellular metabolic substrate utilization is not yet possible due to the limited number of species for which such data are available. We examined substrate preference in the sartorius muscle of the American bullfrog, *Lithobates catesbeiana* [formerly *Rana catesbeiana* (Frost et al., 2006)] and the effect of temperature on the metabolic fate of these substrates.

The metabolic fate of substrate varies between endotherms and ectotherms. Mammalian muscle predominantly oxidizes glucose and fatty acids (Kelley et al., 1988), while lizard (for a review, see Gleeson, 1991), frog (Bennett and Licht, 1974; Fournier and Guderley, 1992) and fish (Pagnotta and Milligan, 1991) muscle rely more heavily on glycolytic exchange between glucose, lactate and glycogen stores for immediate energy supply (for a review, see Gleeson, 1991). Resting concentrations of circulating substrates reflect this preference. Mammals generally are reported to have higher blood glucose than lactate (for a review, see Brooks et al., 1996), while the reverse is true in fish and frogs (Hutchinson and Turney, 1975; Weber et al., 1986; Petersen and Gleeson, 2007). Tuna have higher turnover of labeled carbon from lactate than glucose following activity recovery (Weber et al., 1986). Under most conditions, ectothermy is characterized by high rates of lactate production and utilization for ATP generation and slow rates of oxidation of all substrates. By contrast, muscle from endotherms such as mammals (Brooks and Gaesser, 1980) and flying birds (Jenni-Eirmann et al., 2002) possesses relatively fast rates of oxidative metabolism at rest with preference for glucose (mammals) and fatty acids (birds). Mouse muscle also mainly utilizes glucose for glycogen synthesis (Bonen et al., 1990; Wickler and Gleeson, 1993). From these data, a pattern emerges: (1) active ectotherms rely more heavily on glycolytic pathways than endotherms, (2) endotherms are more likely to completely oxidize substrate and (3) ectotherms also appear to prefer lactate over glucose specifically for glycogen synthesis and in general as a metabolic fuel for skeletal muscle.

Divisions in metabolic substrate utilization between thermoregulatory classes may be correlated to the potent force that temperature exerts on metabolism. Rates of some physical processes, such as diffusion, are nearly temperature independent and thus

demonstrate a $Q_{10}$ rate near 1.0 whereas most biochemical pathways exhibit $Q_{10}$ values upwards of 3.0 (for a review, see Rome et al., 1992). It is clear that rates of muscle metabolism do increase with higher temperature (for a review, see Bennett, 1984). However, it is not known if substrate preference for metabolism is altered by temperature in amphibians.

*Lithobates catesbeiana* is a particularly interesting species of ectotherm in which to investigate not only skeletal muscle substrate preference and use but also how acute and chronic temperature exposure affects this metabolic strategy. This species of frog inhabits a geographic range stretching from sub-arctic to tropical regions (Lannoo, 2005) and can be considered eurythermal, tolerating diurnal changes in pond temperature of up to 30°C (Carey, 1979; A.M.P. and T.T.G., unpublished data). Bullfrogs are large, active predators, and proper allocation of metabolic resources is key to survival in such a wide array of climates. The purpose of the present study was to investigate the effects of acute and acclimatory temperature conditions on substrate preference and substrate metabolic fate in the skeletal muscle of the northern amphibian *L. catesbeiana*. Examining substrate preference and the effects of temperature on these processes may identify patterns of vertebrate muscle fuel utilization in response to different ecological conditions.

## MATERIALS AND METHODS

### Animals and acclimation conditions

Adult male bullfrogs (*Lithobates catesbeiana* Shaw 1802) with a mass of 115–165 g were purchased from Rana Ranch Bullfrog Farm (Twin Falls, ID, USA) and delivered in two equal shipments, in May and July 2007. Upon arrival, frogs were evenly distributed by mass into an acute-exposure group ($N$=6), which served as a control for acclimation changes, and two acclimation groups: cold-acclimated 5°C (CA, $N$=20) and warm-acclimated 25°C (WA, $N$=19). Frogs were housed in communal troughs providing access both to dry areas and water deep enough for submersion. CA frogs were held in an environmental chamber set to 5±2°C with a 12 h:12 h photoperiod for 2–6 weeks. Acute-exposure frogs and WA frogs were held in an environmental chamber set to 25±2°C for 5 days and 2–6 weeks, respectively. WA frogs were fed live crickets twice per week. CA frogs were not fed, as our earlier work and another study have shown that these frogs do not feed or digest at ≤5°C (Riddle, 1909). This protocol was approved by the University of Colorado, Boulder, Institutional Animal Care and Use Committee.

### Muscle bundle preparation

On each day of experiment, one bullfrog was sacrificed by decapitation with a small animal guillotine and double-pithed. The sartorius muscle, with a small portion of tendon on the proximal and distal ends remaining, was carefully dissected from each leg. The muscles were immersed in a modified Ringer solution of 70 mmol $l^{-1}$ NaCl, 2.5 mmol $l^{-1}$ KCl, 1 mmol $l^{-1}$ $MgSO_4$, 1.8 mmol $l^{-1}$ $CaCl_2$ and 25 mmol $l^{-1}$ $NaHCO_3$ in deionized $H_2O$. Each muscle was divided laterally into three fiber bundles, with care taken to avoid severing fibers. Bundles were carefully cleaned of extraneous connective tissue and pinned taut at approximately resting lengths to a Tygon® frame. All bundle dissections were complete within 60 min of removal from the frog. Framed bundles were then placed individually in a 12×75 mm glass incubation chamber containing 3 ml of incubation medium with the addition of 1 mmol $l^{-1}$ D-glucose (J. T. Baker Chemical Company, Phillipsburg, NJ, USA), 5 mmol $l^{-1}$ lactic acid sodium salt (Sigma-Aldrich, St Louis, MO, USA), 1 mmol $l^{-1}$ palmitic acid (Sigma-Aldrich) and 0.4% bovine serum albumin (Sigma-Aldrich). Concentrations of metabolites in the bath reflected resting blood levels of these substrates for anuran amphibians (Hong et al., 1968; Petersen and Gleeson, 2007). Incubation medium also included 1 μCi (37,000 Bq) of one of the following three isotopic labels: D-[U-$^{14}$C]glucose, L-[U-$^{14}$C]lactic acid sodium salt or [1-$^{14}$C]palmitic acid (GE Healthcare UK Limited, Buckinghamshire, UK).

### Temperature conditions

Muscles were prepared and incubated under one of three temperature regimen. Acute exposure bundles were tested at either 5°C, 15°C or 25°C (normal temperature ranges for most bullfrogs in North America). Following the 2–6-week acclimation period, muscles from frogs acclimated to 5°C were tested at either 5°C (acclimation exposure) or 15°C (post-acclimation acute exposure). Muscles from frogs acclimated to 25°C were tested at either 25°C (acclimation exposure) or 15°C (post-acclimation acute exposure). Muscle incubation chambers were maintained at test temperatures by a clear acrylic water jacket flushed with temperature-controlled (±1°C) water from a circulating water bath.

### Incubation conditions

The incubation medium was continuously bubbled at test temperature with approximately 2.1% $CO_2$ and 97.9% $O_2$ for at least 45 min prior to use. This equilibration yielded an incubation pH of 7.9 at 25°C, which is similar to the extracellular pH of resting ranids at this temperature (MacKenzie and Jackson, 1978; Petersen and Gleeson, 2007). The pH of the medium was allowed to fluctuate with temperature as would occur *in vivo* (approximately –0.1 units/+10°C) (MacKenzie and Jackson, 1978). The framed muscle was carefully inserted vertically into the chamber and suspended above a stir bar at the bottom of the chamber. The chamber was capped with a serum stopper with a gas inlet and outlet that allowed for constant infusion of the gas mixture into the airspace at the top of the sealed chamber in order to maintain pH and gas concentration of the medium. The muscle was incubated for 3 h with continuous stirring of the medium by a small stir bar at the bottom of the chamber. We conducted preliminary experiments at room temperature (21±1.5°C) in order to ensure that the muscle was viable under these conditions for the 3 h time period and found that oxygen consumption of the muscle is not different between the first hour of incubation and the third. At the end of the incubation period, the muscle was removed, blotted dry and then rinsed twice in Ringer solution, blotted once more and then submerged in liquid nitrogen until completely frozen. The muscle bundles were then stored for <6 months at –70°C prior to analysis.

### Determination of oxidation to $CO_2$

Immediately following the 3-h incubation, the medium was transferred *via* syringe to a 15 ml flask with a hanging reservoir. The reservoir contained 1 ml of 1:3 ethanolamine:methylcellusolve (Sigma-Aldrich) for sequestration of $CO_2$. 200 μl of 1 mol $l^{-1}$ HCl was added quickly to the chamber, which was then sealed and agitated in order to ensure mixing. The chamber was left for 2 h, at which time the full 1 ml content of the sequestration trap was removed and mixed with Scintiverse scintillation cocktail (Fisher Scientific, Houston, TX, USA) for d.p.m. enrichment determination using a Wallac Model 1204 scintillation counter (Perkin-Elmer, Waltham, MA, USA) with internal quench correction.

### Quantification of intracellular metabolites

Frozen muscle bundles were weighed and then homogenized in six volumes of 6.0% $HClO_4$. Glycogen from the homogenate

supernatant was isolated by ethanol precipitation, and metabolite levels were determined by colorimetric assay, as described previously (Petersen and Gleeson, 2007). This may underestimate any acid-insoluble proglycogen (Asp et al., 1999), although significant amounts of proglycogen in anuran muscle have not yet been reported. Extracellular space corrections were made using an estimate of 20% of muscle wet mass based on the findings of Neville and White (Neville and White, 1979) that incubation conditions increase extracellular space in frog sartorius muscle.

### [$^{14}$C] activity

Muscle bundle homogenate was centrifuged at 7200 ***g*** for 10 min. 150 µl aliquots of supernatant were separated into lactate, glucose and pyruvate fractions by a Dowex 1X8-200 ion-exchange resin column (Sigma-Aldrich) as described previously (Donovan and Gleeson, 2006). Each fraction eluted through the column was then mixed with Scintisafe scintillation cocktail (Fisher Scientific) and counted as above. Initial control experiments ($N$=3) using $^{14}$C-labeled glucose and lactate determined that <0.1% of label was eluted in the inappropriate fraction (e.g. labeled glucose in the lactate fraction), and therefore our column separation of lactate, glucose and pyruvate was considered complete. Aliquots of supernatant were also analyzed for glycogen content and [$^{14}$C]glycogen levels as described previously (Petersen and Gleeson, 2007). Specific activity of [$^{14}$C]glucose, [$^{14}$C]lactate and [$^{14}$C]palmitate were calculated by dividing d.p.m. g$^{-1}$ tissue by total intracellular levels of each metabolite.

Net incorporation rates into glycogen, $CO_2$, pyruvate, and glucose or lactate were calculated by dividing the measured $^{14}$C activity by the specific activity of the intramuscular metabolite, corrected for extracellular space (ECS) $^{14}$C by subtracting the estimated d.p.m. in the ECS (20% of the bundle mass × d.p.m. ml$^{-1}$ incubation medium) from the total d.p.m. measured in the muscle. Extracellular label was assumed to rapidly equilibrate in the tissue, and incorporation rates were assumed to be linear over 3 h of incubation.

StatView 5.01 statistical software was used to conduct data comparisons, with one-way ANOVA and Fisher's PLSD used to determine statistically significant ($P$<0.05) difference between treatment groups.

## RESULTS

Total net uptake and metabolism (Fig. 1) of lactate into the skeletal muscle cell is 5–7× higher than that of glucose ($P$<0.05). Both glucose and lactate net uptake approximately doubles per 10°C increase in temperature ($Q_{10}$ of 2.0), demonstrating that net uptake is a temperature-dependent process for both metabolites. Acute temperature exposure has a marked effect on how glucose is used by the cell (Fig. 2; Table 1). At 5°C, more than 75% of glucose metabolized by the cell is converted to lactate, but this shifts towards glycogen synthesis at warmer temperatures (Fig. 2A). At 25°C, significantly more glucose is oxidized than at lower temperature incubations ($P$<0.05); however, the rate is still extremely low, and is nearly 1/100th of the lactate oxidation rate under the same conditions (Fig. 2A; Table 1).

At 5°C, the major fate for lactate is oxidation (35%), followed by storage as glycogen (25%) and conversion to glucose (25%) (Fig. 2B; Table 1), with a surprisingly large portion also found in pyruvate (15%, data not shown). Higher incubation temperatures result in greater rates of oxidation and a proportional shift in metabolic fate of lactate towards glycogen storage. At both 15°C and 25°C, lactate is mostly stored as glycogen, although a significant portion (20%) of lactate is still oxidized at the higher temperatures (Fig. 2B; Table 1).

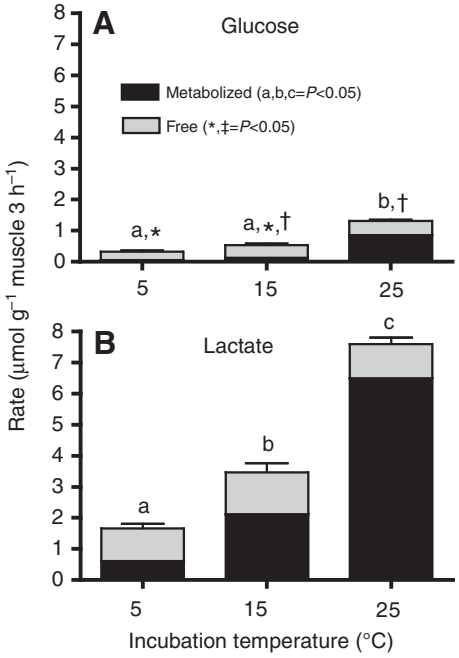


Fig. 1. Net uptake (entire bar, defined as sum of metabolite found to be free, incorporated into glycogen, oxidized, or exchanged in the carbon pool following 3 h of incubation) and metabolism (dark gray, defined as net uptake minus accumulated free pool of metabolite) of glucose (A) and lactate (B) by frog sartorius muscle bundles following 3 h incubation at 5°C, 15°C and 25°C. Differing letters represent significantly different net rates of glucose or lactate metabolized between temperature conditions. Differing symbols represent significantly different net amounts of free intracellular glucose or lactate between temperature conditions ($P$<0.05, one-way ANOVA, Fisher's PLSD, $N$=4–12). Relative proportion of free glucose is highest in 5°C-exposed muscles, while rates of metabolism of both glucose and lactate increase significantly with temperature ($P$<0.05).

Muscles acclimated to 5°C metabolized 30× more lactate than glucose, a factor 3-fold higher than when muscles were exposed acutely to 5°C. Suppression of glucose metabolism at cold-acclimation and incubation temperatures is also suggested by the fact that only 10% of net glucose transported into the cell is metabolized at 5°C, while over 50% of transported lactate is used (Fig. 3).

Following acclimation to 5°C or 25°C, the major fate of glucose was conversion to lactate, and this exchange between the carbon pools is an extremely temperature-sensitive process (Fig. 4; Table 2). Acclimation to 5°C resulted in slightly less than half of the glucose being stored as glycogen, with the majority of glucose converted to lactate. At 25°C this pattern is even more pronounced with nearly 80% of all glucose converted to lactate (Fig. 4A; Table 2). Following acclimation to 5°C, 50% of lactate taken up by the muscle is converted to glucose. By contrast, 25°C warm acclimation results in 85% of intramuscular lactate being stored as glycogen (Fig. 4B; Table 2). Overall, the three fates of glucose examined all demonstrated high temperature sensitivity, with $Q_{10}$ values from 3.6 to 6.3 (Table 2). When tested at a common incubation temperature of 15°C, net glucose uptake and net metabolism did not differ between muscles acclimated to warm and cold conditions (Fig. 5A). By contrast, lactate is influenced significantly by acclimation history (Fig. 5). Threefold more lactate is metabolized than glucose in muscle bundles that were acclimated previously to

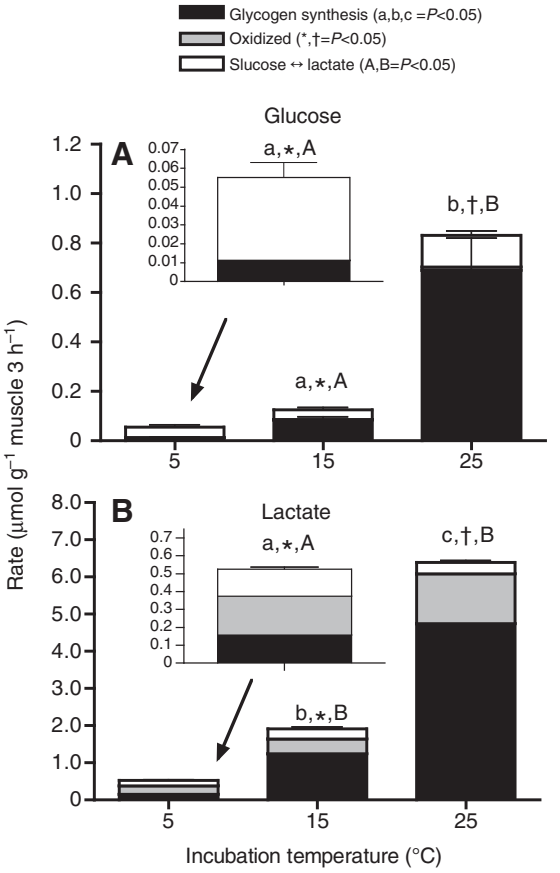


Fig. 2. The effect of acute temperature exposure on the intracellular metabolic fate of glucose (A) and lactate (B) at 5°C (inset), 15°C or 25°C. Differing letters or symbols represent significantly different net rates of glucose or lactate metabolism to glycogen (lowercase letters), oxidized (symbols) or exchange between lactate and glucose carbon pools (uppercase letters) (one-way ANOVA, Fisher's PLSD, $N$=4–12).

a colder temperature, while only 1.3× more lactate was utilized by muscles acclimated previously to a warmer temperature (Fig. 5).

At 15°C, although there is no measurable difference in uptake and net metabolism in glucose, there is a discernible temperature-dependent shift in the fate of metabolized glucose (Fig. 6A). Muscle from frogs acclimated to 5°C stored the majority (70%) of the glucose as glycogen. Muscle from warm-acclimated (25°C) animals demonstrated a reversal of this pattern: significantly more glucose was converted to lactate (70%, $P$<0.05) (Fig. 6A).

At 15°C, cold-acclimated tissue has a higher rate of lactate metabolism than warm-acclimated tissue (Fig. 6, $P$<0.05). Most of the lactate in the cold tissue is converted to glycogen. In warm-acclimated tissue, the metabolic fate of lactate is evenly divided between glycogenesis (glycogen synthesis from glucose) and glyconeogenesis (glycogen synthesis from lactate) (Fig. 6B). Increasing the temperature from 5°C-acclimation conditions to 15°C incubation significantly increases glyconeogenesis as compared with muscles from 25°C-acclimated frogs at the same intermediary temperature of 15°C ($P$<0.05). Unlike the reported response to acute temperature change (Fig. 2), following 2–6 weeks of acclimation to 5 or 25°C, neither glucose nor lactate oxidation rates increase with temperature ($P$>0.05, Fig. 4).

In additional experiments, we measured how oxidation of the free fatty acid palmitate is affected by acute and acclimation temperature regimens. We found that acutely exposing the muscles to 15 or 25°C resulted in significantly higher net rates of palmitate oxidation than incubation at 5°C (Fig. 7A, $P$<0.05). Following acclimation to 5 or 25°C, this pattern persists, as increasing temperature yields significantly higher rates of palmitate oxidation (Fig. 7B, $P$<0.05). Muscles from frogs acclimated to 5°C and incubated at 15°C were found to oxidize significantly less palmitate than muscles acutely exposed to 15°C ($P$<0.05, Fig. 7).

## DISCUSSION

Net uptake of lactate by the muscle cells was 5–7× higher than that of glucose at all temperatures (Fig. 1), demonstrating a strong preference for uptake of lactate by the cell. Net rate of glucose metabolism is more acutely temperature sensitive than that of lactate metabolism. A basic tenet of classical literature on temperature regulation is that certain metabolic processes are 'defended' in the face of temperature change because they are essential to viability of the cell (for reviews, see Guderley, 1990; Hochochka and Somero, 2002). Since net rates of lactate metabolism appear to be less temperature sensitive than net glucose metabolism (Fig. 1; Table 1), our findings support the growing body of literature suggesting that lactate is a more utilized substrate than previously believed (for reviews, see Brooks et al., 1996; Gleeson, 1996; Gladden, 2004).

It should be emphasized that our findings reflect net uptake and utilization of substrate label as assessed at a moment in time after 3 h of incubation. Continuous measurement of flux over time would likely reveal a far more complicated pattern of uptake and utilization than is reflected in the present study and might disclose some futile cycling of label or metabolites that these studies cannot detect. The theoretical sources of error in this approach have been reviewed (Gleeson and Dalessio, 1989) and while they may have some impact on quantitative estimates of net flux under some conditions, likely have little import on the findings of substrate use in this study. Our findings do suggest that under these thermal conditions, lactate is the predominantly metabolized substrate by frog muscle and appears to be the substrate whose utilization is less influenced by temperature.

Studies have demonstrated that lactate is used as a preferred fuel by nerves (Itoh et al., 2003), some oxidative muscles (Andrade and McMullen, 2005), and cardiac tissue under some circumstances (Gertz et al., 1988). We demonstrate here that lactate may also be the preferred substrate in white glycolytic muscle of frogs at rest (Fig. 1; Table 1). Since lactate levels rise by up to 20 mmol $l^{-1}$ following even moderate activity in amphibians (Petersen and Gleeson, 2007), the relative abundance of lactate may be the reason for muscle preference of this substrate. By contrast, blood glucose levels are highly variable and sometimes so low as to be undetectable (Farrar and Frye, 1979; de Roos and Parker, 1982), hence an unreliable substrate source in these animals.

The predominant trend of glucose use by the muscle was glucose conversion to lactate at the lowest temperatures, with increasing storage of glucose as glycogen at higher incubation temperatures (Fig. 2A; Table 1). Since conversion of glucose to lactate (glycolysis) is a major fate at 5°C, glycolysis appears not to be strongly inhibited by acute exposure to cold temperature. Ohira and Ohira report that cold-exposed frog muscle has reduced ATP levels (Ohira and Ohira, 1988), a condition favorable to glycolysis. Trout acutely (48 h) exposed to low temperature demonstrated no reduction in concentrations of glycolytic intermediaries and activators of phosphofructokinase (PFK), fructose-6-phosphate and glucose-6-

Table 1. The effect of acute temperature exposure on metabolic substrate utilization

| | | Glycogen synthesis | | | Oxidation | | | Glucose ↔ lactate conversion | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Incubation $T^{\circ}$ | Rate | $N$ | $Q_{10}$ | Rate | $N$ | $Q_{10}$ | Rate | $N$ | $Q_{10}$ |
| Glucose | | | | | | | | | | |
| | 5 | 0.011±0.003[a] | 6 | | 0.0004±0.0003[a] | 6 | | 0.044±0.0080[a] | 6 | |
| | | | | 7.6 | | | 5.0 | | | 0.9 |
| | 15 | 0.084±0.011[a] | 8 | | 0.0020±0.0006[a] | 8 | | 0.038±0.0106[a] | 7 | |
| | | | | 8.2 | | | 7.0 | | | 3.3 |
| | 25 | 0.690±0.128[b] | 12 | | 0.0138±0.0470[b] | 11 | | 0.128±0.0155[b] | 12 | |
| Lactate | | | | | | | | | | |
| | 5 | 0.158±0.050* | 5 | | 0.216±0.062* | 5 | | 0.151±0.013* | 5 | |
| | | | | 7.8 | | | 1.8 | | | 1.7 |
| | 15 | 1.245±0.252† | 4 | | 0.398±0.080† | 4 | | 0.264±0.051† | 4 | |
| | | | | 3.8 | | | 3.4 | | | 1.1 |
| | 25 | 4.753±0.221‡ | 4 | | 1.335±0.355† | 4 | | 0.300±0.019† | 4 | |

Values are rate (μmol $g^{-1}$ muscle 3 $h^{-1}$) of glucose or lactate incorporation into glycogen, oxidation or the exchange between substrate pools ± s.e.m. Differing symbols (lactate) and differing letters (glucose) represent a significant difference in metabolic fate of glucose or lactate between temperature exposure groups ($P$<0.05, one-way ANOVA, Fisher's PLSD)

bisphospate (Lehoux and Guderley, 1997), suggesting minimal inhibition of ectothermic muscle glycolysis by acute exposure to cold temperature. Maintenance of pathways of lactate formation at low temperature would seem beneficial to frogs, as lactate could then be utilized as the substrate throughout the body during variable ambient temperature conditions (for a review, see Gladden, 2004).

Under all acute temperature conditions, exchange occurs between lactate, glycogen and glucose pools, as evidenced by our finding that a small, but measurable, percentage of lactate carbon was found in the form of free glucose (Fig. 2; Table 1). We report here that significant amounts of carbon from labeled lactate, up to 25% of labeled lactate in fact, are measured in the form of glucose at the end of the 3-h incubation (Fig. 2; Table 1), which would be consistent with the provocative findings of Fournier and Guderley that muscle may be a net producer of glucose (Fournier and Guderley, 1992;

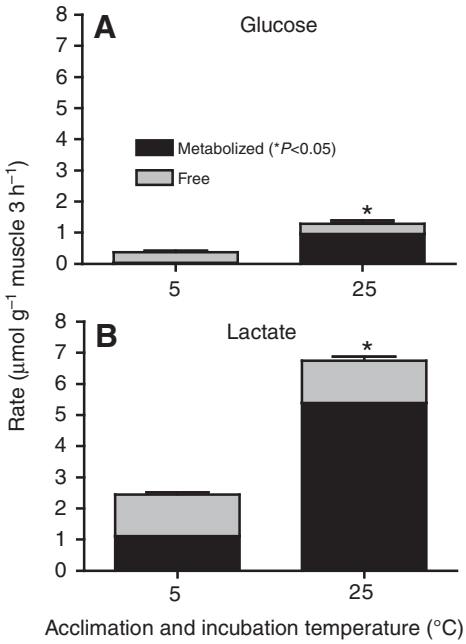


Fig. 3. Net uptake (entire bar) and metabolism (dark gray) of glucose (A) and lactate (B) by frog sartorius muscle bundles following acclimation to 5°C or 25°C. Muscles were incubated at their respective acclimation temperature. * Net rate of metabolism is significantly different between temperature treatment groups ($P$<0.05, one-way ANOVA, Fisher's PLSD, $N$=4–11). Warm acclimation and incubation conditions yield higher net metabolism of lactate than glucose in frog muscle ($P$<0.05).

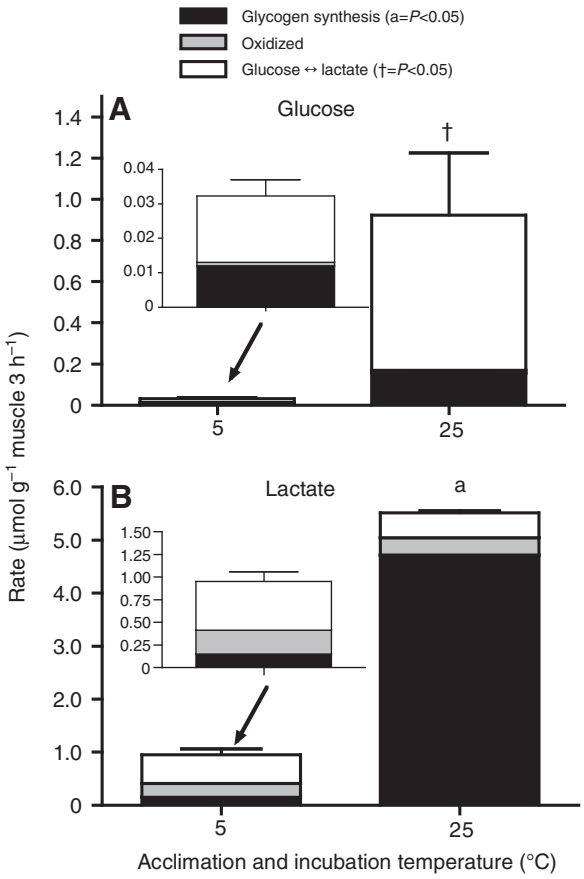


Fig. 4. The effect of temperature acclimation and incubation on the intracellular metabolic fate of glucose (A) and lactate (B) at 5°C (inset) or 25°C in sartorius muscle. Muscles were incubated at their respective acclimation temperatures. Letters and symbols represent a significant effect of temperature condition on glycogen synthesis (a), or conversion between lactate and glucose (†) by lactate or glucose ($P$<0.05, one-way ANOVA, Fisher's PLSD, $N$=4–11). Warm acclimation and incubation results in an increased conversion of glucose to lactate and increased glycogen synthesis from lactate ($P$<0.05).

Table 2. The effect of acclimation temperature exposure on metabolic substrate utilization

| | Acclimation/ Incubation $T$° | Glycogen synthesis | | | Oxidation | | | Glucose ↔ lactate conversion | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Rate | $N$ | $Q_{10}$ | Rate | $N$ | $Q_{10}$ | Rate | $N$ | $Q_{10}$ |
| Glucose | | | | | | | | | | |
| | 5/5 | 0.012±0.002[a] | 6 | | 0.001±0.000[a] | 6 | | 0.019±0.005[a] | 6 | |
| | 5/15 | 0.858±0.128[b] | 8 | | 0.028±0.008[b] | 8 | | 0.366±0.075[a,b] | 8 | |
| | 25/15 | 0.249±0.079[a,c] | 4 | | 0.029±0.006[b] | 4 | | 0.78±0.212[b] | 4 | |
| | 25/25 | 0.155±0.024[a] | 5 | 3.6 | 0.014±0.003[a,b] | 5 | 3.7 | 0.755±0.303[b] | 4 | 6.3 |
| Lactate | | | | | | | | | | |
| | 5/5 | 0.152±0.019* | 10 | | 0.262±0.125 | 10 | | 0.539±0.109 | 11 | |
| | 5/15 | 2.481±0.316† | 7 | | 0.574±0.287 | 6 | | 0.956±0.474 | 7 | |
| | 25/15 | 0.655±0.158*,‡ | 4 | | 0.142±0.044 | 4 | | 0.649±0.170 | 4 | |
| | 25/25 | 4.716±0.295** | 7 | 5.6 | 0.333±0.077 | 7 | 1.1 | 0.464±0.041 | 7 | 0.9 |

Values are rate (μmol $g^{-1}$ muscle 3 $h^{-1}$) of glucose or lactate incorporation into glycogen, oxidation, or the exchange between substrate pools ± s.e.m. Differing symbols (lactate) and differing letters (glucose) represent a significant difference in metabolic fate of glucose or lactate between temperature exposure groups ($P$<0.05, one-way ANOVA, Fisher's PLSD). $Q_{10}$ values represent temperature sensitivity of average rate change between 5°C and 25°C.

Fournier and Guderley, 1993a; Fournier and Guderley, 1993b). These findings are in keeping with those of Fournier et al. (Fournier et al., 1994), who reported that even when frogs were hepatectomized, whole-body glucose, lactate and glycogen metabolism were unaltered, suggesting that the muscle may be able to produce and even export glucose.

Cold-acclimated muscles metabolized 30× more lactate than glucose, a factor 3× as high as when muscles were exposed acutely to 5°C, suggesting that one aspect of cold acclimation may be increased capacity to metabolize lactate, but not glucose (Fig. 3). Disproportionate suppression of glucose *vs* lactate metabolism at low temperature ($Q_{10}$ of 15.0 *vs* 2.4; Table 2) suggests that long-term cold-temperature acclimation inhibits glycolysis much more than oxidative or gluconeogenic pathways (Fig. 3). Such a case could occur following acclimation to low temperature if there was long-term, cold-induced, inhibition of muscle PFK or pyruvate kinase (PK), known regulators of glycolysis. There is evidence for inhibition of activity of PFK at cold temperature in bird muscle (Marsh and Dawson, 1982), although studies in hibernating squirrels (MacDonald and Storey, 2001) and at least one species of fish (Lehoux and Guderley, 1997) suggest that temperature may have

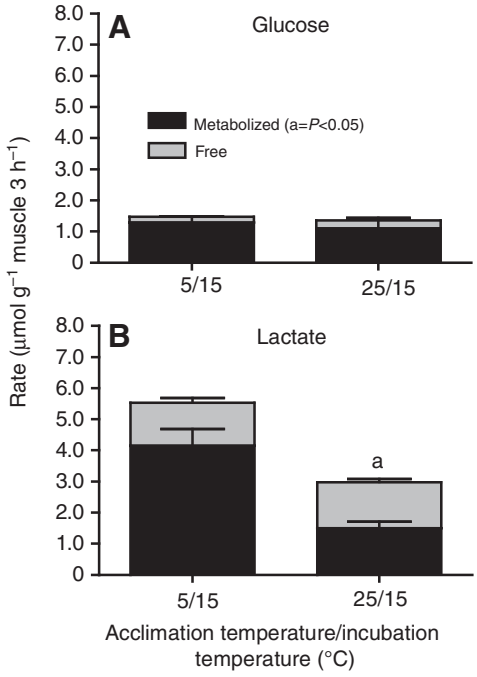


Fig. 5. Net uptake (entire bar) and metabolism (dark gray) of glucose (A) and lactate (B) by frog sartorius muscle bundles following acclimation at 5 or 25°C and 3 h incubation at a common temperature of 15°C. (a) Net rate of lactate metabolism at 15°C is significantly lower in sartorius muscles from frogs acclimated to 25°C ($P$<0.05, one-way ANOVA, Fisher's PLSD, $N$=4–11).

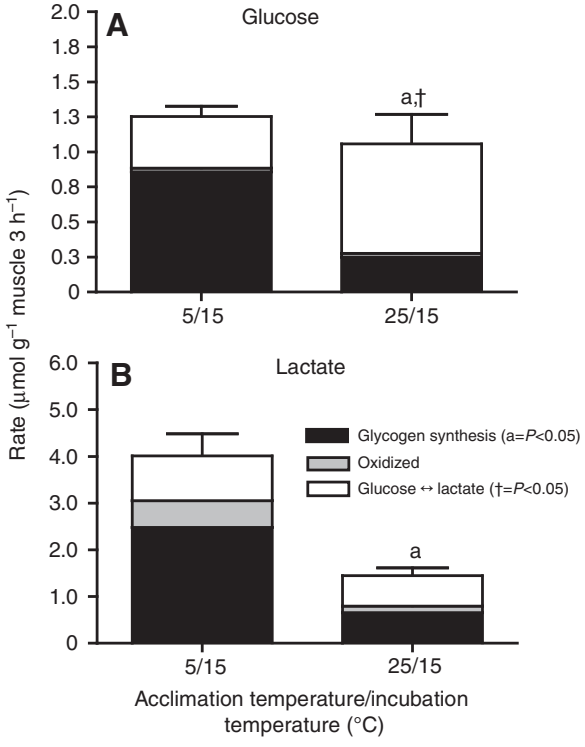


Fig. 6. The effect of temperature acclimation and incubation on the intracellular metabolic fate of glucose (A) and lactate (B) in sartorius muscles from frogs acclimated to 5 or 25°C and incubated at the common temperature of 15°C. Letters and symbols represent a significant effect of temperature condition on net glycogen synthesis (a) or conversion between lactate and glucose (†) ($P$<0.05, one-way ANOVA, Fisher's PLSD, $N$=4–12).

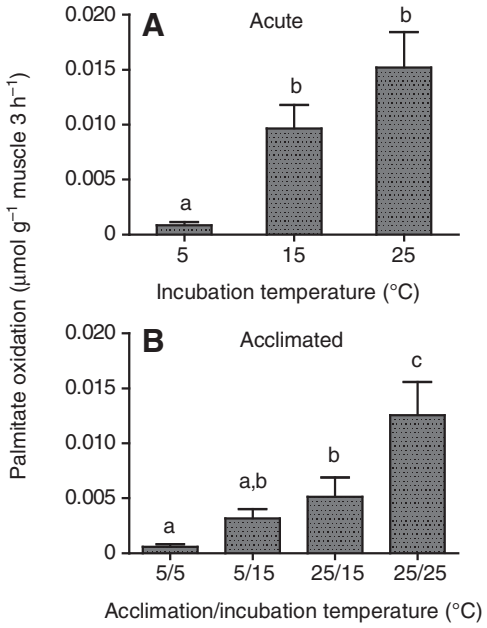


Fig. 7. The effect of acute (A) and acclimation (B) temperature exposure on palmitate oxidation in frog sartorius muscle. Differing letters indicate a significant difference in rate of palmitate oxidation between temperature treatments ($P$<0.05, one-way ANOVA, Fisher's PLSD, $N$=5–7). Increasing incubation temperature both acutely and following acclimation causes an increase in palmitate oxidation ($P$<0.05).

only a limited effect on PFK activity and may be compensated for by long-term acclimation (Guderley and Gawlicka, 1992).

Muscles from frogs acclimated to 25°C had a significantly higher conversion rate of glucose to lactate at the higher temperatures and demonstrated significant sensitivity to temperature change (Fig. 4A). Consistent with this finding, $Q_{10}$ values for glucose-to-lactate conversion are the highest of any process we measured (Table 2). It is possible that activity of lactate dehydrogenase (LDH) increases sharply with warm temperature acclimation, as shown in other ectotherms (Feder, 1983; Seebacher and James, 2008). Following 4 months of hibernation, LDH activity is decreased in at least one species of frog (St Pierre and Boutillier, 2001). A study from the fish literature proposes that at cold temperature, LDH concentrations and kinetics are altered, in order to favor a larger pyruvate pool for protection of aerobic potential (Zackhartsev et al., 2004). Our findings that less glucose is converted to lactate following cold acclimation could support this observation.

Prior acclimation temperature had no significant effect on net rate of glucose uptake or metabolism at 15°C (Fig. 4A). Similarly, striped bass acclimated to 5°C and 25°C also demonstrated no compensation in red or white muscle tissues for glucose use but did appear to upregulate pathways of palmitate oxidation (Jones and Sidell, 1982).

Metabolic compensation did, however, occur in terms of lactate metabolism (Fig. 5B). Lactate was metabolized at 2× the rate by cold-acclimated muscles incubated at 15°C than warm-acclimated muscles at the same temperature (Fig. 5B). This pattern is in keeping with the 'classic' concept of metabolic compensation in which low-temperature acclimation results in higher enzyme binding affinity, activity levels or concentration, resulting in higher disposal rates for specific substrates (for a review, see Hochachka and Somero, 2002). In the present study, increased lactate metabolism occurred in bullfrog muscle in the absence of changes in glucose utilization. This suggests to us that one central aspect of frogs' response to cold is an increased utilization of lactate as a metabolic fuel (Fig. 5; Table 1).

Lactate glyconeogenesis is enhanced in muscles from frogs acclimated to cold temperature. Since bullfrogs (Stinner et al., 1994), and other ranids (Cunjack, 1986), are 'cold active' underwater in winter, it may be ecologically important during cold seasons for muscle tissues to be able to quickly store substrate that becomes available following activity. Gleeson suggests that ectotherms in general utilize glycogen storage as a depot for lactate produced following activity, allowing muscles to dispose of accumulated lactate while replenishing glycogen stores for future use (Gleeson, 1991). Fig. 6 is suggestive of enhancement of glycogenic and glyconeogenic pathways, which would be a logical strategy if food sources were scarce and energy metabolism overall is reduced, as would be the case while overwintering.

Prior to overwintering conditions, ranid frogs are known to build up lipid stores, in the form of fat bodies (Fitzpatrick, 1976), and increase glycogen storage in the liver (Byrne and White, 1975). Our data do not support a substantial role for fat oxidation in resting muscle under our conditions (Fig. 7). It does not appear that palmitate oxidation constitutes a large component of energy metabolism in these resting muscle cells. Temperature does have a significant effect on oxidation rates. However, acclimation does not alter this pattern and no compensatory adjustments appear to exist to increase palmitate oxidation following acclimation to a colder temperature (Fig. 7B).

We report here that lactate metabolism is of central importance to amphibian muscle cells, with net rates of metabolism of this substrate far exceeding rates of glucose or palmitate use. Acute exposure to cold temperature results in most glucose being converted to lactate while both substrates are stored mostly as glycogen at warm temperature. Acute exposure to cold temperature also reduces glucose metabolism more markedly than lactate metabolism, signifying a role for lactate as a staple fuel under these conditions. We suggest that ready use of lactate as a substrate in general, and enhancement of glyconeogenic pathways specifically, could play a key role in the tolerance of this species to temperature fluctuation by allowing for storage of available substrate during cold-induced low metabolism.

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