Kinematic performance and muscle activation patterns during
post-freeze locomotion in wood frogs (*Rana sylvatica*)

Javier H. Santos-Santos^{1,2*},

Brett M. Culbert³,

Emily M. Standen³

¹Department of Animal Biology, University of Barcelona, Avenida
Diagonal 645, 08028, Barcelona, Spain

²Department of Biogeography and Global Change, Museo Nacional de
Ciencias Naturales - CSIC, Calle Jose Gutierrez Abascal 2, 28006, Madrid,
Spain

³Department of Biology, University of Ottawa, Ottawa, Canada

*corresponding author: J.H. Santos-Santos, jssantos@mncn.csic.es

Abstract

Wood frogs [*Rana sylvatica* (LeConte 1825)] exhibit one of the most extreme freeze tolerance responses found in vertebrates. While extensive work is continuing to resolve the physiological mechanisms involved, few have studied the effects of freezing on locomotor performance. The ability to mount an appropriate locomotor response is vital, as locomotion can affect both survivorship and reproductive success. To investigate how the biomechanical processes during locomotion are altered following freezing, stroke cycle timings and kinematic performance were measured prior to, and immediately following a freeze-thaw cycle. Additionally, the effects of cooling rate (0.3°C/h vs. 0.8°C/h) were also assessed. While jumping and swimming performance were both reduced post-freeze, the effects were more pronounced during swimming, with observed reductions in velocity and distance travelled. Interestingly, these changes occurred largely independent of cooling rate. Altered stroke cycle timings and highly variable muscle activation/deactivation patterns suggest an impairment in muscle function as frogs continued to recover from the effects of freezing. This was supported by the physiology of frogs post-freeze, specifically, the persistence of elevated glucose levels in muscles important during locomotion. Collectively, these findings suggest that reductions in locomotor performance observed immediately following a freeze-thaw cycle are driven by alterations in muscle function.

Keywords: wood frog, *Rana sylvatica*, freeze tolerance, cryoprotectants, cooling rate, biomechanics, locomotion

1. Introduction

Many animals cope with seasonally subzero ($< 0^{\circ}\text{C}$) temperatures; as such, a diverse suite of strategies to facilitate survival during these periods have evolved. Many animals seek thermal refugia, and may even enter prolonged periods of inactivity and reduced metabolism (*i.e.* hibernation) to overcome these seasonal hardships (Sømme 1989; Heggenes et al. 1993; Terrien et al. 2011; Williams et al. 2015). However, ectotherms arguably possess the most impressive overwintering strategies of all animals. Many ectotherms, including insects, reptiles, and amphibians synthesize large quantities of cryoprotectant compounds (*e.g.* glucose and glycerol) prior to the onset of subzero temperatures (Storey and Storey 1988). Cryoprotectants allow animals to avoid freezing by reducing the temperature at which ice crystals begin to form in tissues—allowing them to function at subzero temperatures (Storey 1997). However, some ectotherms have taken this a step further.

Certain species can tolerate freezing (Storey and Storey 1987; Storey and Storey 1988); relying largely on amassing high levels of cryoprotectants in tissues to discourage damage (Storey and Storey 1992). These effects are amplified via dehydration of tissues, which further increases cryoprotectant concentrations (Lee et al. 1992; Churchill and Storey 1993). During this period animals enter a frozen state where breathing rate, heart rate, and blood flow cease (Layne et al. 1989; Layne and First 1991). Survival during subzero periods depends on a variety of factors, including cooling rate, freezing temperature, and the frequency of subzero episodes (Layne and Lee 1987; Costanzo et al. 1992). Of all freeze tolerant vertebrate species, wood frogs [*Rana sylvatica* (LeConte 1825)] have been the most thoroughly studied.

Like all freeze tolerant species, wood frogs rely on a combination of tissue dehydration and cryoprotectant synthesis to cope with freezing. In anticipation of freezing, muscle proteolysis causes an increase in urea production, while liver glycogen stores are rapidly converted to glucose immediately upon initiation of freezing—elevating levels of both cryoprotectant molecules (Costanzo et al. 2015). Similarly, as a result of an increased reliance on anaerobic metabolism during both freezing and dehydration, lactate levels rise (Churchill and Storey 1993). However, upon thaw wood frogs reconvert the majority of the glucose mobilized as cryoprotectant back into hepatic glycogen reserves (Storey and Storey 1986; Costanzo et al. 2013), providing energy stores to facilitate behaviours such as mating.

Wood frogs begin to perform intense spawning behaviours immediately upon spring arousal (Wells and Bevier 1997). However, when competing with controls that were not frozen, recently thawed male wood frogs are less successful at achieving amplexus with a female (Costanzo et al. 1997). As well, locomotor endurance remains impaired for at least 96h following thaw (Irwin et al. 2003). These combined findings suggest that despite the urge to perform and reproduce immediately upon thaw, an altered physiological condition is likely preventing them from doing so. In fact, Layne and Rice (2003) observed that jump distance and swim speed were negatively affected 6h post-freeze, only returning to pre-freeze levels after 54h. However, no studies to date have assessed how kinematics during locomotion are altered in recently thawed frogs.

We hypothesized that biomechanical performance in *R. sylvatica* post-thaw is reduced due to prolonged effects of the widespread physiological remodeling induced during freezing. To assess this, we compared the kinematics and muscle activation patterns of

R. sylvatica before and after freezing, and measured several physiological parameters implicated in their freeze tolerance response. In addition, we assessed the effect of the rate of cooling, predicting that more abrupt decreases in temperature would cause further reductions in biomechanical performance. The effects of holding time on animal performance and condition were also assessed.

2. Materials and Methods

2.1. Collection and Housing

Fifty-one male wood frogs (*Rana sylvatica*) were used in this study. Animals were collected on April 1, 2016 in Bishop Mills, Ontario. Following collection, animals were transferred to holding containers at the University of Ottawa. Containers were kept in the dark at 4°C, and contained one centimeter of dechloraminated City of Ottawa tap water. Water was changed every three days. Wood frogs were not fed over the duration of the study to avoid the potentially lethal effects due to unpredictable ice nucleation occurring in the contents of the gut while freezing (Storey and Storey 1987). All animals were checked to be sexually mature (*i.e.* inspection of nuptial pads) and in good condition (*i.e.* completely unfrozen and uninjured) prior to experimentation. All experimental protocols were approved by the University of Ottawa animal care committee (protocol BL2548), and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

2.2. Experimental Procedure

All experimental procedures (*i.e.* holding, transportation, anesthesia, operation, recovery, and filming) were performed at 4°C. Experiments were conducted from April 8-24, 2016. Six non-jumped controls (twelve in total) were randomly sampled from holding on the first and last days of the experiment to assess changes in tissue metabolite concentrations over time. Each experimental cycle (four in total) included nine frogs and lasted four days (Table 1).

Treatments consisted of a slow cooling regime, where the environmental temperature was reduced from 4°C to -2.5°C over a 24h period; a rapid cooling regime, where the environmental temperature was reduced from 4°C to -2.5°C over an 8h period; and a sham treatment, where muscles were prodded without electrode insertion to control for any effects of surgery and animals were not frozen (Table 1). The order of treatment assignment was determined randomly prior to retrieving the animals for surgery. Animal temperatures were monitored throughout the cooling regime using a RDXL4SD 4-channel data logger thermometer equipped with four type T thermocouples (OMEGA), and freezing exotherms were observed during both cooling regimes.

On Day 1, frogs were randomly collected from holding and underwent surgery (see Section 2.2.1). Following surgery, animals were given 24h to recover at 4°C. On Day 2, video recordings of jumping and swimming performance were taken (see Section 2.2.2). Following filming, animals were returned to their containers and placed into their respective cooling treatment. Sham-handled animals were filmed, and immediately euthanized and dissected. On Day 3, animals in the rapid cooling treatment were placed at 4°C for 8h. Previous work has shown that sciatic nerve excitability and hind limb retraction returns within this time range (Kling et al. 1994). Following this, animals were filmed for a second time, and immediately euthanized and dissected. On Day 4, the same protocol described for Day 3 was performed on animals from the slow cooling treatment.

2.2.1. Surgery

Following collection from holding, animals were weighed, measured (snout-vent length (SVL)), and placed on a covered bed of ice. Bi-filament stainless steel wire electrodes

(0.002 μm ; California Fine Wire Co.) were surgically implanted into the gastrocnemius and semimembranosus muscles using a 26 gauge needle (Figure 1). These muscles were selected due to their ubiquitous importance during locomotion. Electrodes were sutured together at the knee, ischium, and sacrum along the right side of the animal to prevent animal injury and damage to the wires. Over the duration of the surgery, the temperature of the animal, and its surrounding materials, were monitored using a temperature data logger with a type K thermocouple (OMEGA). After surgery, animals were placed in chilled water to recover. Animals were then individually put into containers with moist sphagnum moss and placed at 4°C. The lid of each container was modified to allow electrode movement to prevent injury to both the animals and the wires. Animals of each regime were placed in separate incubators (Precision M815; Thermo Scientific) at 4°C.

2.2.2. Filming

Two high-speed video cameras (FASTCAM Mini UX100; Photron) were fixed around an 80L aquarium, containing 40L of water. The lateral camera was fixed at the same plane as the water level, while the ventral camera reflected off of a mirror positioned at a 45° angle underneath the aquarium. The tank was insulated and water chilled to 4°C. Water temperature was monitored using a temperature data logger with a type K thermocouple throughout each trial.

Animals were placed on moist sphagnum moss on top of a brick embedded in ice, which served both to maintain water temperature and as a jumping platform that impeded the frog from swimming backwards. Ice bricks were replaced between treatments. The temperature of each animal was periodically monitored up until the

point of jumping using a type K thermocouple. Each animal jumped and swam between two and four times, which was filmed at 500 frames per second.

2.2.3. Electromyography

The electromyography (EMG) electrodes were connected to P511 Grass amplifiers (Natus Medical Inc.). Amplifiers and camera trigger were connected to a PowerLab DAQ (ADInstruments Inc.). Synchronization of high-speed video and EMG electrode data was performed via an end trigger through the PowerLab DAQ with the LabChart (v.8.1.5) interface at a 10kHz sampling rate and a $\pm 5V$ differential signal range. A 60Hz notch filter was applied to remove potential alternating current electrical noise. Our baseline signal was 0.05V. Onset and offset were determined manually in MATLAB at approximately 0.3-0.5V.

2.2.4. Dissections

Immediately following final video recordings, animals were rapidly euthanized via double pithing. Mass and length (SVL) were recorded, and blood samples were collected via aortic severance into heparinized capillary tubes. Tubes were centrifuged at 10,000g for 3 minutes and hematocrit was measured. Plasma was extracted, flash frozen in liquid nitrogen, and stored at $-80^{\circ}C$ for later analysis of glucose, lactate, and urea. The liver, right gastrocnemius, and right semimembranosus were extracted, weighed, and flash frozen in liquid nitrogen. Hepatosomatic index was calculated as the percentage of the liver in relation to the mass of the entire animal. These samples were stored at $-80^{\circ}C$ for later analysis of glycogen, glucose, lactate, and urea. To calculate relative muscle water content, the right peroneus muscle was extracted, weighed, placed into an incubator at $65^{\circ}C$ for 30 minutes, and reweighed. The percent difference

between these weights represented relative water content. During dissection, proper placement of the EMG electrodes was confirmed. All dissections were performed on ice.

2.3. Tissue Preparation and Metabolite Measurements

Tissue samples were ground using a mortar and pestle on dry ice prior to analysis. Glucose/glycogen, lactate, and urea levels were determined according to the methods described by Keppler and Decker (1974), Brandt et al. (1980), and Rahmatullah and Boyd (1980), respectively. Analyses of glucose, glycogen, and lactate were performed in triplicate; however, urea was run in duplicate due to limited sample volumes. Inter- and intra-assay variations (% CV) for glucose (Inter, Intra; 6.2%, 4.4%), glycogen (1.7%, 6.1%), lactate (3.0%, 3.7%), and urea (6.1%, 9.7%) were all below 10%.

2.4. Data Processing

Single view high-speed videos were digitized in MATLAB (DLTdv5; Hedrick 2008; Release 2016a; MathWorks). In swimming videos, six anatomical landmarks were tracked in the ventral view: (A) tip of the nose; (B) ischium; (C) femur head; (D); ankle; (E) tarsus; and (F) tip of the second (longest) phalange (Figure 1). Animal trajectories calculated from the ischium and four angles (foot, ankle, knee, and hip) were measured to describe the motion of the leg following Peters et al. (1996; Figure 1). The stroke cycle began with extension of the foot, and finished following the completion of foot flexion. In jumping videos, the tip of the nose was tracked in the lateral view to calculate jump height and distance. A calibration cube was used to convert pixel lengths to distance measurements in both views.

2.4.1. Kinematics

Peak joint extension and flexion were determined by plotting calculated angles over time. Average jump and swim velocity were calculated as the total distance covered in each trial divided by time, while instantaneous velocity was calculated at every 4th frame (*i.e.* 8 ms). Distance and velocity during kick and recovery correspond to the foot extension and flexion phases, respectively, of each stroke cycle. The jump loading time was calculated as the time from the visible flexing of the stationary frog's epaxial musculature (see beginning movement point (BM) (Peters et al. 1996)) to the first visible motion of leg extension. At this point the animal is in a characteristic position of an upward head/snout tilt; a stance that immediately leads to hind limb extension. Swimming was categorized as either synchronous, asynchronous, or both—based on the observed initiation of leg extension. Stroke cycle timings were calculated using polar coordinates so that individual stroke cycles could be compared. Peak joint angle magnitude timings, as well as EMG muscle activation timings, were transformed into stroke cycle polar coordinates in radians.

2.5. Statistical Analyses

All data are presented as means $\pm$ SEM. Unpaired *t*-tests were used to assess whether animal physiology or kinematic locomotor performance at the end of the experiment (*i.e.* Day 16) varied from that of animals at the beginning of the experiment (*i.e.* Day 1). One-way analysis of variance was employed to detect differences in physiology and kinematic performance between cycles within treatments. Unpaired *t*-tests were used to evaluate differences between rapid and slow cooling regimes for all physiological variables, and to compare with sham-handled controls. Paired *t*-tests were employed to assess differences between pre- and post-freeze locomotor performance. For variables

with more than one measure per animal, values were averaged and means were analyzed. When data did not meet the assumptions of homoscedasticity and normality, analyses were performed using transformed data. Variables that showed a von Mises distribution and equal variance were tested for directionality using Rayleigh's test. If variables are directional that means they occur at the same time in every stroke cycle. Analyses of kinematic and physiological data were performed in SigmaPlot (Version 11, Systat), while cycle timings were analyzed in MATLAB (Release 2016a; MathWorks). A significance level of 0.05 was used for all analyses.

3. Results

3.1. Does holding duration affect physiology or locomotor performance?

When comparing the physiological characteristics of non-jumped control frogs sampled directly from holding at the beginning of the experiment (Day 1) to those sampled at the end of the experiment (Day 16), several differences were observed (Table 2A). Lactate levels in both the gastrocnemius ($p=0.020$) and semimembranosus ($p<0.001$) increased over the duration of the experiment, along with plasma ($p=0.030$) and semimembranosus ($p<0.001$) glucose concentrations. Muscle water content ($p=0.004$) increased over this period as well. Plasma lactate ($p<0.001$), hepatic glucose ($p=0.001$), and semimembranosus glycogen ($p=0.030$) levels all decreased across time.

Locomotor performance of jumped sham-handled control frogs did not vary across time (Day 1 vs. Day 16) for any of the kinematic variables assessed (Table 2B).

3.2. Does cooling regime influence physiology?

Upon scrutiny of the data, no differences were detected for any of the kinematic or physiological variables across the four experimental cycles for any of the treatment groups. Thus, data were pooled across cycles within each treatment group for the remaining analyses.

Animals differed very little physiologically between the two cooling regimes (Table 3A); the lone difference being frogs from the rapid regime exhibited higher liver glycogen concentrations ($p=0.030$). However, when comparing pooled frogs from both cooling regimes to sham-handled control frogs, several differences were observed (Table 3B). Glucose concentrations of previously frozen frogs were elevated in the

plasma ($p=0.002$), liver ($p=0.006$), gastrocnemius ($p<0.001$), and the semimembranosus ($p<0.001$). Lactate levels were reduced in the gastrocnemius ($p=0.001$) and the semimembranosus ($p<0.001$) of previously frozen frogs. Frogs from the slow cooling regime also displayed lower hepatic glycogen levels than sham-handled controls ($p<0.001$), however, rapidly cooled frogs did not differ from controls ($p=0.240$).

3.3. How does locomotor performance change following freeze?

Frogs from the slow cooling regime (Table 4A) displayed reductions in distance kicked ($p=0.040$), took longer to enter the jumping position ($p=0.020$), and had larger maximum flexion angles at the foot ($p=0.040$) post-freeze. Rapidly cooled frogs (Table 4B) exhibited reductions in mean jump velocity ($p<0.001$), kick velocity ($p=0.030$), and maximum instantaneous swimming velocity ($p=0.040$), as well as performing asynchronous swimming more frequently ($p=0.027$) following freeze. To further investigate the effects of freezing on locomotor performance, we analyzed the before and after measures of experimental animals irrespective of treatment (Table 5). Overall, frogs displayed reductions in average jumping velocity ($p=0.007$), average swimming velocity ($p=0.013$), kick distance ($p=0.025$), kick velocity ($p=0.004$), maximum instantaneous swimming velocity ($p=0.007$), and took longer to enter a jumping position ($p=0.004$) following freeze.

Effects of freezing on cycle timing occurred for both cooling regimes (Figure 2). Frogs from the rapid cooling regime (Table 6A) reached their maximum flexion angle at the foot later ($p=0.020$), while performing their maximum extension angles at the ankle ($p=0.049$) and hip ($p=0.030$) earlier in the stroke cycle. Frogs from the slow cooling regime, on the other hand, reached their maximum extension angles at the foot

($p<0.001$), ankle ($p=0.005$), and knee ($p=0.030$) later in the stroke cycle, as well as their maximum flexion angle at the ankle ($p=0.003$) earlier (Table 6B).

Following the slow cooling regime, muscle activation timings of the both the gastrocnemius and the semimembranosus were highly variable and did not occur at a particular time during the stroke cycle (non-directional; Raleigh's test ($p>0.05$); Table 6A). Similarly, the muscle activation of the semimembranosus in rapidly cooled frogs was also non-directional. The gastrocnemius of rapidly cooled frogs did occur at the same point in each stroke cycle (directional; Raleigh's test ($p<0.05$)). Rapidly cooled frogs did not display changes in either the start ($p=0.500$) or end ($p=0.520$) of activation in the gastrocnemius as a consequence of freezing (Table 6B).

4. Discussion

Physiology

Wood frogs undergo drastic physiological remodeling to tolerate freezing. As such, it is not surprising that frogs which underwent freezing had different physiological responses than non-frozen sham-handled controls. Elevated levels of glucose along with low levels of hepatic glycogen in previously frozen animals suggests that the resorption of this cryoprotectant molecule occurs over a longer duration than the 8 hour recovery period provided. Indeed, prior studies have shown that the effects of freezing on glucose metabolism persist even following 5 days of recovery from freezing at 4°C (Costanzo et al 2013). As well, consistent with previous findings, this universal rise in glucose occurred independently of muscle glycogen stores (Storey 1984), instead relying solely on hepatic glycogen levels (Table 3B). The functional outcome of this being that glycogen stores remain high in muscle tissues where they are required for high intensity locomotor and reproductive behaviours. Interestingly, there were almost no physiological differences between frogs that were cooled at different rates (Table 3A), suggesting that the physiological effect of cooling rate is minor when compared to the broad responses induced by freezing in general.

Lactate is known to accumulate while frozen (Storey and Storey 1984), owing to an increased reliance on anaerobic metabolism. Lactate levels negatively correlate with muscle performance in frogs (Fitts and Holloszy 1976), therefore, it would be feasible to expect that elevated lactate would be associated with reductions in post-freeze locomotor performance (Layne and Rice 2003; current study). However, in contrast, we observed lower levels of muscle lactate in previously frozen animals compared to sham-handled controls (Table 3B), and previous studies suggest that either lactate does not

accumulate in the leg muscles (Costanzo et al. 1997), or that it is rapidly cleared upon thaw (<24hr; Irwin et al. 2003). In support of the latter, Sinclair et al. (2013) observed that whole animal metabolic rate increases during thaw, probably due in part to lactate clearance. This is supported by elevated post-freeze activity levels of lactate dehydrogenase (Cowan and Storey 2001), which converts lactate to pyruvate. Overall, these data suggest that lactate is not the cause of the reduction in locomotor performance observed post-thaw.

Muscle atrophy induced while frozen may also cause reduced locomotor performance, however, immobility while frozen does not seem to cause muscle atrophy in wood frogs (Irwin et al. 2003; Layne and Rice 2003). More likely, reductions in creatine phosphate (PCr) levels that occur as a result of freezing may be influencing performance (Storey and Storey 1984). PCr stores are relied upon by frogs while frozen to maintain constant levels of adenosine triphosphate, and thus become depleted (Storey and Storey 1984; Layne and Kennedy 2002). However, PCr is also used by anurans as a fuel source during periods of high intensity muscle activation, such as burst locomotion (Miller and Sabol 1989). Therefore, it is likely that decreased PCr levels are, at least partly, responsible for the observed reductions in post-freeze locomotor performance. We also observed elevated levels of glucose in both the gastrocnemius and the semimembranosus muscles (Table 3B)—a persisting artifact of the prior freeze response. It is possible that excess of this cryoprotectant molecule may have further inhibitory effects on muscle function and thus locomotor performance in anurans, and future work should investigate this.

Layne and Rice (2003) found that lower freezing temperatures required longer recovery periods before pre-freeze levels of locomotor performance returned. While we did not vary freezing temperatures in the present study, we did vary the rate of temperature decline. Costanzo et al. (1992) reported significant differences in organ dehydration and glucose content due to cooling rate, with both being impaired in frogs cooled at a rate of -1.6°C/h compared to -0.2°C/h . The rapid cooling rate used in the present study was $\sim 50\%$ less than that used in Costanzo et al. (1992), however, previous work has shown that cooling rates $\leq -1.0^{\circ}\text{C/h}$ are associated with high rates of mortality (Costanzo et al. 1991). While no mortalities as a result of freezing were observed in the current study, we also did not detect any differences in glucose levels indicative of impaired glucose mobilization, despite hepatic glycogen levels being higher in rapidly cooled frogs. It is therefore likely that cooling rates necessary to impair glucose mobilization in this species are lethal.

In contrast to earlier studies (Layne 1992; Costanzo et al. 1997) that required a 24h thaw at 3°C for wood frogs to respond by jumping, frogs from both of our cooling regimes recovered jumping and swimming ability within 8h thawing at 4°C . That our frogs recovered coordinated motor function sooner than those of earlier studies (southern Ohio spring emergers) could be due to intraspecific differences originating from their postglacial colonization biogeography, however, divergence between wood frog populations' may already predate the last glacial maximum. Lee-Yaw et al. (2008) defined two distinct mitochondrial lineages (*i.e.* Eastern and Western) expanding from two different glacial refugia. Our frogs' lineage (Western lineage), which has colonized from Alaska to the Great Lakes region, may have inherited superior freeze-tolerance mechanisms than those from southern Ohio (Eastern lineage) due to the lower

temperatures encountered at higher latitudes. This is supported by the fact that genetics appear to have a strong effect on the thermal physiology of wood frogs (Manis and Claussen 1986), and that morphological variation reflects the genetic differences between lineages (Martof and Humphries 1959; Lee-Yaw et al. 2008).

Biomechanics

While frogs in the current study underwent significant physiological changes as a result of holding over the 16 day period of experiments (Table 2A), their kinematic performance measures were unaffected (Table 2B). The lack of quantifiable change in biomechanical performance hints at the resilience of the frog musculoskeletal system to function effectively at a broad range of physiological parameters.

Frogs that underwent freezing, however, displayed reduced kinematic performance. Previous work shows that freezing dramatically decreases *in vitro* muscle function in wood frogs up to 24h following thaw (Layne 1992), and reduces locomotor performance even following 11 days of recovery (Layne and Rice 2003). These findings have important ecological implications, as wood frogs experience highly variable conditions over the winter, potentially undergoing dozens of repeated freeze-thaw cycles (Sinclair et al. 2013). This not only increases their odds of being preyed upon, but given that wood frogs spawn immediately after thawing (Wells and Bevier 1997), a reduction in locomotor performance may lead to a reduction in reproductive success.

The decrease in locomotor performance suggests a decrease in muscle output and, thus, a less explosive locomotor event. This hypothesis is partially supported by changes observed in kinematic and muscle activation patterns of treatment animals. For

example, in slowly cooled animals, leg extension starts earlier and ends later in the stroke cycle, occupying a larger portion of the stroke cycle via a slower motion pattern (Figure 2A). Rapidly cooled animals did not exhibit this extended power phase, but rather they delayed their knee extension, while accelerating extension of their hip and ankle (Figure 2B)—a seemingly variable pattern suggestive of decreased function and control. Of more direct interest is the change in the rotation of the foot during swimming. Richards (2008) showed that foot rotation of a swimming frog is an important mechanism affecting thrust production. The position of the foot during the recovery stroke is also essential for reducing drag during flexion. If this timing is altered, the foot is not streamlined and can increase drag, explaining the decreases in swimming velocity and distance travelled.

During this study, we also measured muscle activation timing in the gastrocnemius and the semimembranosus muscles. Both muscles flex the knee, while the semimembranosus also extends the hip and the gastrocnemius flexes the foot. Therefore, both muscles are important in successful thrust production during jumping and swimming. Wood frogs recover hind limb muscle contractility within 1-2h post-thaw, however, coordinated motor function and body posturing remains impaired for 14-24h (Layne and Lee 1995; Costanzo et al. 1997). During this time, processes such as muscle fiber recruitment may be suboptimal, affecting locomotor performance. In the current study, we observed highly variable EMG timings for both the gastrocnemius and the semimembranosus muscles of the leg following freezing (Tables 6A and 6B). Also notable, frogs from the rapid cooling treatment exhibited an increase in asynchronous kicking behavior when swimming post-freeze. This behavioral modification has performance consequences and may be a result of post-freeze neuromotor control

impairment (Kling et al. 1994); frogs that activate all joints synchronously during swimming produce higher thrust forces (Nauwelaerts and Aerts 2003; Johanssen and Lauder 2004).

Jumping performance does not appear to be as affected as swimming performance post-freeze (Table 5). This difference in response may be the result of the mechanical differences between forces produced on land vs. in water. Muscle forces produced during jumping are transferred into more effective ground reaction forces compared with the more lateral production of forces during swimming (Nauwelaerts and Aerts 2003). In addition, elastic energy storage may differ between terrestrial and aquatic locomotion such that loading of elastic elements during jumping is more effective than during swimming (Aerts and Nauwelaerts 2009). If ground reaction forces and elastic storage are more important during jumping, impaired muscle function may not be as detrimental to jump performance as it is to swim performance, which relies more directly on muscle contraction and contraction rate.

Over the course of the experiment we observed rehydration of the leg muscles and reductions in circulating lactate levels in our frogs (Table 2A), changes consistent with recovering from freezing. Given that we collected these animals in April, immediately following spring emergence, these changes suggest that the frogs in the current study may still have been recovering from the effects of winter freezing. Both reduced muscle pH (Renaud and Stevens 1984) and muscle dehydration (Preest and Pough 1989) negatively affect contractile function in the muscles of anurans, suggesting that our frogs may have already exhibited muscle impairment prior to experimental freezing. While kinematic performance did not vary over the duration of the experiment (Table

2B), future studies should investigate whether the degree of biomechanical resilience varies seasonally (*i.e.* spring vs. fall).

Acknowledgements

This work was supported by a NSERC Discovery Grant and a Canadian Foundation for Innovation Grant to EMS, and an ERASMUS MUNDUS – NOVA DOMUS CHEMEDPHO Action 2 Grant to JHSS. We also acknowledge the University of Barcelona and the University of Ottawa for their collaboration in the ERASMUS MUNDUS framework financed by the European Commission. Many thanks are extended to Vance Trudeau, Michal Galus, Maria Vu, and Brad Weiler for assistance with frog collection and care, Nicolas Corradi for allowing us to use his incubators, Kathleen Gilmour for letting us use her plate reader and reagents, Riley Jones for performing the urea analysis, and the many undergraduate students who assisted with conducting experiments, performing surgeries, processing tissues, and digitizing videos.

Bibliography

- Aerts, P., and Nauwelaerts, S. 2009. Environmentally induced mechanical feedback in locomotion: frog performance as a model. *J. Theor. Biol.* **261**(3): 372–378.
- Brandt, R.B., Siegel, S.A., Waters, M.G., and Bloch, M.H. 1980. Spectrophotometric assay for D-(-)-lactate in plasma. *Anal. Biochem.* **102**(1): 39–46.
- Churchill, T.A., and Storey, K.B. 1993. Dehydration tolerance in wood frogs: a new perspective on development of amphibian freeze tolerance. *Am. J. Physiol. Integr. Comp. Physiol.* **265**(6): R1324–R1332.
- Costanzo, J.P., do Amaral, M.F.C., Rosendale, A.J., and Lee, R.E. 2013. Hibernation physiology, freezing adaptation and extreme freeze tolerance in a northern population of the wood frog. *J. Exp. Biol.* **216**(18): 3461–3473.
- Costanzo, J.P., Irwin, J.T., and Lee, R.E. 1997. Freezing impairment of male reproductive behaviors of the freeze-tolerant wood frog, *Rana sylvatica*. *Physiol. Zool.* **70**(2): 158–166.
- Costanzo, J.P., Lee, R.E., and Wright, M.F. 1992. Cooling rate influences cryoprotectant distribution and organ dehydration in freezing wood frogs. *J. Exp. Biol.* **261**(4): 373–378.
- Costanzo, J.P., Lee, J. R, and Wright, M.F. 1991. Effect of cooling rate on the survival of frozen wood frogs, *Rana sylvatica*. *J. Comp. Physiol. B.* **161**: 225–229.
- Costanzo, J.P., Reynolds, A.M., Clara, M., Do Amaral, F., Rosendale, A.J., and Lee, R.E. 2015. Cryoprotectants and extreme freeze tolerance in a subarctic population of the wood frog. *PLoS One*, **10**(2): 3461–3473.
- Cowan, K.J., and Storey, K.B. 2001. Freeze-thaw effects on metabolic enzymes in wood frog organs. *Cryobiology*, **43**(1): 32–45.

- Fitts, R., and Holloszy, J. 1976. Lactate and contractile force in frog muscle during development of fatigue and recovery. *Am. J. Physiol.* **231**(2): 430–433.
- Hedrick, T.L. 2008. Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinspir. Biomim.* **3**: 034001 (6pp).
- Heggenes, J., Krog, O.M.W., Lindås, O.R., Dokk, J.G., Lindas, O.R., and Dokk, J.G. 1993. Homeostatic behavioural responses in a changing environment: brown trout (*Salmo trutta*) become nocturnal during winter. *J. Anim. Ecol.* **62**(2): 295–308.
- Irwin, J.T., Costanzo, J.P., and Lee, R.E. 2003. Postfreeze reduction of locomotor endurance in the freeze tolerant wood frog, *Rana sylvatica*. *Physiol. Biochem. Zool.* **76**(3): 331–338.
- Johansson, L.C., and Lauder, G.V. 2004. Hydrodynamics of surface swimming in leopard frogs (*Rana pipiens*). *J. Exp. Biol.* **207**(22): 3945–3958.
- Keppler, D., and Decker, K. 1974. Methods for determination of metabolites. I. Carbohydrate metabolites: glycogen determination with amyloglucosidase. *In* *Methods of Enzymatic Analysis. Edited by H.U. Bergmeyer.* Academic Press, New York. pp. 1127–1131.
- Kling, K.B., Costanzo, J.P., and Lee, R.E. 1994. Post-freeze recovery of peripheral nerve function in the freeze-tolerant wood frog, *Rana sylvatica*. *J. Comp. Physiol. B.* **164**(4):316-320.
- Layne, J.R. 1992. Postfreeze survival and muscle function in the leopard frog (*Rana pipiens*) and the wood frog (*Rana sylvatica*). *J. Therm. Biol.* **17**(2): 121–124.
- Layne, J.R., and First, M.C. 1991. Resumption of physiological functions in the wood frog (*Rana sylvatica*) after freezing. *Am. J. Physiol.* **261**(1): R134–R137.

- Layne, J.R., and Kennedy, S.D. 2002. Cellular energetics of frozen wood frogs (*Rana sylvatica*) revealed via NMR spectroscopy. *J. Therm. Biol.* **27**(3): 167–173.
- Layne, J.R., and Lee, R.E. 1987. Freeze tolerance and the dynamics of ice formation in wood frogs (*Rana sylvatica*) from Southern Ohio. *Can. J. Zool.* **65**(8): 2062–2065.
- Layne, J.R., and Lee, R.E. 1995. Adaptations of frogs to survive freezing. *Clim. Res.* **5**:53-59.
- Layne, J.R., Lee, R.E., and Heil, T.L. 1989. Freezing-induced changes in the heart rate of wood frogs (*Rana sylvatica*). *Am. J. Physiol.* **257**(5): R1046–R1049.
- Layne, J.R., and Rice, M.E. 2003. Postfreeze locomotion performance in wood frogs (*Rana sylvatica*) and spring peepers (*Pseudacris crucifer*). *Can. J. Zool.* **81**(12): 2061–2065.
- Lee, R.E., Costanzo, J.P., Davidson, E.C., and Layne, J.R. 1992. Dynamics of body water during freezing and thawing in a freeze-tolerant frog (*Rana sylvatica*). *J. Therm. Biol.* **17**(4–5): 263–266.
- Lee-Yaw, J.A., Irwin, J.T., and Green, D.M. 2008. Postglacial range expansion from northern refugia by the wood frog, *Rana sylvatica*. *Mol. Ecol.* **17** (3): 867-884.
- Manis, M.L., and Claussen, D.L. 1986. Environmental and genetic influences on the thermal physiology of *Rana sylvatica*. *J. Therm. Biol.* **11**(1): 31–36.
- Martof, B.S., and Humphries, R.L. 1959. Geographic variation in the wood frog, *Rana sylvatica*. *Am. Midl. Nat.* **61**(2): 350–389.
- Miller, K., and Sabol, J.L. 1989. The role of phosphocreatine breakdown in the metabolic support of locomotion by clawed frogs, *Xenopus laevis*. *Comp. Biochem. Physiol. B.* **93**(2): 251–254.

- Nauwelaerts, S., and Aerts, P. 2003. Propulsive impulse as a covarying performance measure in the comparison of the kinematics of swimming and jumping in frogs. *J. Exp. Biol.* **206**(23): 4341–4351.
- Peters, S.E., Kamel, L.T., and Bashor, D.P. 1996. Hopping and swimming in the leopard frog, *Rana pipiens*. I. Step cycles and kinematics. *J. Morphol.* **230**(1): 1–16.
- Preest, M.R., and Pough, F.H. 1989. Interaction of temperature and hydration on locomotion of toads. *Funct. Ecol.* **3**(6): 693–699.
- Rahmatullah, M., and Boyde, T.R. 1980. Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin. Chim. Acta*, **107**(1–2): 3–9.
- Renaud, J.M., and Stevens, E.D. 1984. The extent of short-term and long-term compensation to temperature shown by frog and toad sartorius muscle. *J. Exp. Biol.* **75**(1): 57–75.
- Richards, C.T. 2008. The kinematic determinants of anuran swimming performance: an inverse and forward dynamics approach. *J. Exp. Biol.* **211**(19): 3181–3194.
- Sinclair, B.J., Stinziano, J.R., Williams, C.M., Macmillan, H.A., Marshall, K.E., and Storey, K.B. 2013. Real-time measurement of metabolic rate during freezing and thawing of the wood frog, *Rana sylvatica*: implications for overwinter energy use. *J. Exp. Biol.* **216**(2): 292–302.
- Sømme, L. 1989. Adaptations of terrestrial arthropods to the alpine environment. *Biol. Rev. Camb. Philos. Soc.* **64**(4): 367–407.
- Storey, K.B. 1984. Freeze tolerance in the frog, *Rana sylvatica*. *Experientia*, **40**(11): 1261–1262.

- Storey, K.B. 1997. Organic solutes in freezing tolerance. *Comp. Biochem. Physiol. A.* **117**(3): 319–326.
- Storey, K.B., and Storey, J.M. 1984. Biochemical adaption for freezing tolerance in the wood frog, *Rana sylvatica*. *J. Comp. Physiol. B.* **155**(1): 29–36.
- Storey, K.B., and Storey, J.M. 1986. Freeze tolerant frogs: cryoprotectants and tissue metabolism during freeze-thaw cycles. *Can. J. Zool.* **64**: 49-56.
- Storey, K.B., and Storey, J.M. 1987. Persistence of freeze tolerance in terrestrially hibernating frogs after spring emergence. *Copeia*, 1987(3): 720–726.
- Storey, K.B., and Storey, J.M. 1988. Freeze tolerance in animals. *Physiol. Rev.* **68**(1): 27–84.
- Storey, K.B., and Storey, J.M. 1992. Natural freeze tolerance in ectothermic vertebrates. *Annu. Rev. Physiol.* **54**(54): 619–637.
- Terrien, J., Perret, M., and Aujard, F. 2011. Behavioral thermoregulation in mammals: a review. *Front. Biosci.* **16**: 1428–1444.
- Wells, K.D., and Bevier, C.R. 1997. Contrasting patterns of energy substrate use in two species of frogs that breed in cold weather. *Herpetologica*, **53**: 70–80.
- Williams, C.M., Henry, H.A.L., and Sinclair, B.J. 2015. Cold truths: How winter drives responses of terrestrial organisms to climate change. *Biol. Rev. Camb. Philos. Soc.* **90**(1): 214–235.

Figure 1. Schematic representation of a wood frog (*Rana sylvatica*). Six anatomical landmarks were digitized in the ventral view to calculate animal movement. The expanded region in the dorsal view shows the position of the two muscles examined in this study. The m. semimembranosus is a hip extensor and knee flexor that originates on the ischium and inserts on the medial tibiofibula. The m. gastrocnemius originates posteriorly on the femoral condyles and inserts on the plantar aponeurosis posterior of the tibiotarsal joints acting as a foot flexor, and also flexes the leg at the knee joint. Single bipolar electromyography electrodes were implanted into each muscle to measure activation patterns during jumping and swimming.

Figure 2. Timing of kinematic variables for the right leg of wood frogs (*Rana sylvatica*) during swimming before (thick grey) and after (thin black) freeze. One complete stroke cycle is represented by 360 degrees; 0 degrees represents the start of extension and 180 degrees the start of flexion. **A)** Timing of leg extension and flexion before and after the slow cooling treatment. **B)** Timing of leg extension and flexion before and after the rapid cooling treatment. **C)** EMG muscle activation/deactivation timing differences before and after the rapid cooling treatment. Significant differences before and after treatment are denoted with an asterisk.

Figure 1. Schematic representation of a wood frog (*Rana sylvatica*). Six anatomical landmarks were digitized in the ventral view to calculate animal movement. The expanded region in the dorsal view shows the position of the two muscles examined in this study. The m. semimembranosus is a hip extensor and knee flexor that originates on the ischium and inserts on the medial tibiofibula. The m. gastrocnemius originates posteriorly on the femoral condyles and inserts on the plantar aponeurosis posterior of the tibiotarsal joints acting as a foot flexor, and also flexes the leg at the knee joint. Single bipolar electromyography electrodes were implanted into each muscle to measure activation patterns during jumping and swimming.

169x157mm (300 x 300 DPI)

Figure 2. Timing of kinematic variables for the right leg of wood frogs (*Rana sylvatica*) during swimming before (thick grey) and after (thin black) freeze. One complete stroke cycle is represented by 360 degrees; 0 degrees represents the start of extension and 180 degrees the start of flexion. A) Timing of leg extension and flexion before and after the slow cooling treatment. B) Timing of leg extension and flexion before and after the rapid cooling treatment. C) EMG muscle activation/deactivation timing differences before and after the rapid cooling treatment. Significant differences before and after treatment are denoted with an asterisk.

202x224mm (300 x 300 DPI)

Table 1. Experiment cycle timeline and procedures. Columns denote cooling treatments and sample sizes. Four 4-day experiment cycles were run in total.

DAY	CONTROLS N=3	RAPID COOLING N=3	SLOW COOLING N=3
1	Surgery: sham (20min.)	Surgery: electrodes (30min.)	Surgery: electrodes (30min.)
	Recovery @ 4°C (24h)	Recovery @ 4°C (24h)	Recovery @ 4°C (24h)
2	Filming (30min.)	Filming (30min.)	Filming (30min.)
	Euthanized and Dissected	Cooling 4°C to -2.5°C (over 8h)	Cooling 4°C to -2.5°C (over 24h)
		Frozen @ -2.5°C (12h)	
3		Thaw @ 4°C (8h)	
		Filming (30min.)	Frozen @ -2.5°C (12h)
		Euthanized and Dissected	
4			Thaw @ 4°C (8h)
			Filming (30min.)
			Euthanized and Dissected

Table 2A. Differences in physiological measures of non-jumped control wood frogs (*Rana sylvatica*) over the duration of the experiment. Frogs were sampled directly from their holding tanks on Days 1 and 16. Data are presented as means $\pm$ the standard error of the mean (SEM) and significant results are shown in **bold**.

Tissue		N		Day 1	Day 16	t	p
		Day 1	Day 16	Mean $\pm$ SEM	Mean $\pm$ SEM		
Glucose ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	6	5	3.70 $\pm$ 0.75	13.42 $\pm$ 4.09	2.57	0.030
	Liver	6	6	13.18 $\pm$ 1.21	5.65 $\pm$ 1.22	4.38	0.001
	Semimembranosus	6	6	2.06 $\pm$ 0.20	9.38 $\pm$ 0.34	18.59	<0.001
	Gastrocnemius	6	6	1.92 $\pm$ 0.29	1.24 $\pm$ 0.15	2.06	0.700
Glycogen ($\mu\text{mol mL}^{-1}$ or g^{-1})	Liver	6	6	30.85 $\pm$ 2.12	23.50 $\pm$ 5.99	1.16	0.270
	Semimembranosus	6	6	2.76 $\pm$ 0.50	1.39 $\pm$ 0.19	2.59	0.030
	Gastrocnemius	6	6	3.01 $\pm$ 0.60	1.78 $\pm$ 0.30	1.83	0.100
Lactate ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	6	5	6.45 $\pm$ 0.82	1.57 $\pm$ 0.34	5.13	<0.001
	Liver	6	6	2.74 $\pm$ 0.25	2.37 $\pm$ 0.33	0.91	0.390
	Semimembranosus	6	6	10.5 $\pm$ 0.85	26.08 $\pm$ 0.84	13.02	<0.001
	Gastrocnemius	6	6	5.91 $\pm$ 0.66	8.96 $\pm$ 0.80	2.94	0.020
Urea ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	6	6	6.94 $\pm$ 0.64	6.09 $\pm$ 0.67	0.92	0.380
	Liver	6	6	3.31 $\pm$ 0.21	3.32 $\pm$ 0.50	0.01	0.990
	Semimembranosus	6	6	3.61 $\pm$ 0.30	3.02 $\pm$ 0.14	1.79	0.100
	Gastrocnemius	6	6	2.97 $\pm$ 0.12	3.04 $\pm$ 0.12	0.45	0.660
Hepatosomatic Index (%)		6	6	3.62 $\pm$ 0.34	2.97 $\pm$ 0.23	1.59	0.140
Muscle H ₂ O Content (%)		6	6	37.48 $\pm$ 1.49	47.80 $\pm$ 2.35	3.71	0.004
Hematocrit (%)		6	6	30.50 $\pm$ 3.14	19.15 $\pm$ 4.62	2.03	0.070

Table 2B. Differences in kinematic measures of sham-handled control wood frogs (*Rana sylvatica*) over the duration of the experiment. Data are presented as means ± the standard error of the mean (SEM). No significant differences were detected. Angles are reported in radians.

	Variable	N		Day 1	Day 16	t	p
		Day 1	Day 16	Mean ± SEM	Mean ± SEM		
Jumping	Distance (mm)	3	6	123.03 ± 12.15	199.22 ± 37.93	1.36	0.220
	Avg Velocity (cm sec ⁻¹)	3	5	26.65 ± 5.40	31.04 ± 4.67	0.60	0.570
	Max Height (mm)	3	6	0.06 ± 0.06	16.53 ± 7.21	1.56	0.160
	Time to Jump (msec)	3	6	14.67 ± 3.53	9.33 ± 0.67	2.13	0.070
Swimming	Avg Velocity (cm sec ⁻¹)	3	5	26.65 ± 5.40	31.04 ± 4.67	0.60	0.570
	Kick Distance (mm)	3	5	54.91 ± 11.33	78.71 ± 15.07	1.10	0.320
	Kick Velocity (cm sec ⁻¹)	3	5	38.66 ± 7.42	46.35 ± 6.54	0.75	0.480
	Distance Recovery (mm)	3	5	29.57 ± 5.38	57.09 ± 8.30	2.34	0.060
	Velocity Recovery (cm sec ⁻¹)	3	5	18.34 ± 2.49	23.02 ± 2.31	1.31	0.240
	Max Instantaneous Velocity (cm sec ⁻¹)	3	5	46.89 ± 7.72	65.04 ± 10.23	1.23	0.270
Maximum Extension Angles	Foot	3	5	3.17 ± 0.01	3.09 ± 0.05	1.33	0.230
	Ankle	3	5	3.07 ± 0.06	3.06 ± 0.05	0.11	0.920
	Knee	3	5	2.60 ± 0.06	2.56 ± 0.06	0.39	0.710
	Hip	3	5	2.56 ± 0.07	2.77 ± 0.06	2.12	0.080
Maximum Flexion Angles	Foot	3	5	4.17 ± 0.15	4.20 ± 0.07	0.22	0.840
	Ankle	3	5	0.92 ± 0.04	0.83 ± 0.08	0.82	0.440
	Knee	3	5	0.24 ± 0.07	0.19 ± 0.05	0.61	0.560
	Hip	3	5	1.63 ± 0.01	1.48 ± 0.07	1.50	0.190

Table 3A. Physiological differences between wood frogs (*Rana sylvatica*) from the slow and rapid cooling regimes. Data are presented as means $\pm$ the standard error of the mean (SEM). Significant results are shown in **bold**.

Tissue		<i>N</i>		Slow Cooling	Rapid Cooling	<i>t</i>	<i>p</i>
		Slow Cooling	Rapid Cooling	Mean $\pm$ SEM	Mean $\pm$ SEM		
Glucose ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	11	12	25.77 $\pm$ 2.95	25.55 $\pm$ 5.11	0.90	0.380
	Liver	12	12	25.27 $\pm$ 2.69	25.11 $\pm$ 3.40	0.04	0.970
	Semimembranosus	12	12	9.29 $\pm$ 0.74	10.27 $\pm$ 1.30	0.03	0.890
	Gastrocnemius	12	12	10.78 $\pm$ 0.75	11.49 $\pm$ 1.61	0.34	0.740
Glycogen ($\mu\text{mol mL}^{-1}$ or g^{-1})	Liver	12	10	8.33 $\pm$ 2.43	18.50 $\pm$ 3.71	2.367	0.030
	Semimembranosus	12	12	3.05 $\pm$ 0.40	2.52 $\pm$ 0.32	1.22	0.240
	Gastrocnemius	12	12	3.15 $\pm$ 0.42	2.11 $\pm$ 0.35	1.93	0.070
Lactate ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	11	12	10.13 $\pm$ 0.86	9.34 $\pm$ 0.72	0.79	0.490
	Liver	12	12	3.48 $\pm$ 0.31	4.18 $\pm$ 0.23	1.81	0.080
	Semimembranosus	12	12	8.09 $\pm$ 1.15	9.19 $\pm$ 0.75	0.80	0.430
	Gastrocnemius	12	12	2.99 $\pm$ 0.18	2.68 $\pm$ 0.18	1.26	0.220
Urea ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	11	12	6.69 $\pm$ 0.49	6.90 $\pm$ 0.91	0.15	0.880
	Liver	12	12	3.57 $\pm$ 0.24	3.41 $\pm$ 0.38	0.68	0.510
	Semimembranosus	12	12	3.65 $\pm$ 0.25	3.21 $\pm$ 0.19	1.43	0.170
	Gastrocnemius	12	12	2.99 $\pm$ 0.18	2.68 $\pm$ 0.18	1.19	0.250
Hepatosomatic Index (%)		12	12	2.80 $\pm$ 0.18	2.90 $\pm$ 0.19	0.36	0.720
Muscle H ₂ O Content (%)		11	12	38.08 $\pm$ 2.20	40.49 $\pm$ 3.04	0.59	0.570
Hematocrit (%)		11	12	24.57 $\pm$ 2.23	25.29 $\pm$ 1.17	0.29	0.770

Table 3B. Physiological differences between combined treatment and sham-handled control wood frogs (*Rana sylvatica*). Liver glycogen was compared separately for each cooling regime as a difference was detected between cooling regimes (see Table 3A). Data are presented as means ± the standard error of the mean (SEM). Significant results are shown in **bold**.

Tissue		N		Treatments	Controls	t	p
		Treatments	Controls	Mean ± SEM	Mean ± SEM		
Glucose ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	23	15	25.65 ± 2.95	10.44 ± 3.61	3.26	0.002
	Liver	24	15	25.19 ± 2.12	16.04 ± 2.04	2.92	0.006
	Semimembranosus	24	15	9.78 ± 0.74	5.21 ± 0.81	4.04	<0.001
	Gastrocnemius	24	14	11.13 ± 0.87	3.31 ± 0.38	6.63	<0.001
Glycogen ($\mu\text{mol mL}^{-1}$ or g^{-1})	Liver	Slow-12	15	8.33 ± 2.43	23.81 ± 2.61	4.25	<0.001
		Rapid-10		18.50 ± 3.71		1.21	0.240
	Semimembranosus	24	15	2.79 ± 0.25	2.30 ± 0.26	1.27	0.210
	Gastrocnemius	24	15	2.63 ± 0.29	2.16 ± 0.29	1.10	0.280
Lactate ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	23	15	9.72 ± 0.55	7.96 ± 0.96	1.71	0.090
	Liver	24	15	3.83 ± 0.20	4.54 ± 0.35	1.92	0.060
	Semimembranosus	24	15	8.64 ± 0.68	19.83 ± 1.56	7.48	<0.001
	Gastrocnemius	24	15	8.96 ± 0.91	14.32 ± 1.23	3.57	0.001
Urea ($\mu\text{mol mL}^{-1}$ or g^{-1})	Plasma	23	14	6.80 ± 0.52	6.62 ± 0.44	0.24	0.810
	Liver	24	15	3.49 ± 0.22	3.25 ± 0.21	0.73	0.470
	Semimembranosus	24	15	3.43 ± 0.16	3.25 ± 0.14	0.80	0.430
	Gastrocnemius	24	15	2.84 ± 0.13	2.87 ± 0.17	0.14	0.890
Hepatosomatic Index (%)		24	15	2.85 ± 0.13	2.69 ± 0.15	0.81	0.420
Muscle H ₂ O Content (%)		23	13	39.34 ± 1.88	42.53 ± 2.94	0.96	0.350
Hematocrit (%)		23	15	24.95 ± 1.20	25.34 ± 1.87	0.19	0.850

Table 4A. Effects of the slow cooling regime on wood frog (*Rana sylvatica*) kinematics. Data are presented as means $\pm$ the standard error of the mean (SEM). Significant results are shown in **bold**. Angles are reported in radians.

	Variable	<i>N</i>	Before Mean $\pm$ SEM	After Mean $\pm$ SEM	<i>t</i>	<i>p</i>
Jumping	Distance (mm)	12	161.41 $\pm$ 25.67	170.15 $\pm$ 28.31	0.23	0.820
	Avg Velocity (cm sec ⁻¹)	12	56.04 $\pm$ 7.59	49.54 $\pm$ 8.30	0.53	0.610
	Max Height (mm)	12	4.28 $\pm$ 3.77	8.75 $\pm$ 5.81	0.63	0.540
	Jump Loading Time (msec)	12	23.33 $\pm$ 5.25	59.83 $\pm$ 11.15	2.80	0.020
Swimming	Avg Velocity (cm sec ⁻¹)	8	29.08 $\pm$ 3.76	17.45 $\pm$ 3.15	2.18	0.060
	Kick Distance (mm)	8	74.45 $\pm$ 10.18	42.17 $\pm$ 4.70	2.54	0.040
	Kick Velocity (cm sec ⁻¹)	8	44.49 $\pm$ 6.81	25.92 $\pm$ 5.00	2.14	0.070
	Distance Recovery (mm)	8	48.61 $\pm$ 6.95	46.61 $\pm$ 6.67	0.01	0.990
	Velocity Recovery (cm sec ⁻¹)	8	20.98 $\pm$ 2.46	13.81 $\pm$ 2.55	1.75	0.120
	Max Instantaneous Velocity (cm sec ⁻¹)	8	56.74 $\pm$ 7.74	34.10 $\pm$ 6.31	2.03	0.080
Maximum Extension Angles	Foot	8	3.05 $\pm$ 0.04	3.08 $\pm$ 0.03	0.47	0.650
	Ankle	8	2.88 $\pm$ 0.04	2.91 $\pm$ 0.05	0.55	0.600
	Knee	8	2.37 $\pm$ 0.06	2.32 $\pm$ 0.06	1.04	0.330
	Hip	8	2.63 $\pm$ 0.06	2.55 $\pm$ 0.09	0.75	0.480
Maximum Flexion Angles	Foot	8	4.42 $\pm$ 0.10	4.65 $\pm$ 0.09	2.47	0.040
	Ankle	8	0.94 $\pm$ 0.08	0.83 $\pm$ 0.09	0.08	0.940
	Knee	8	0.21 $\pm$ 0.03	0.24 $\pm$ 0.07	0.41	0.690
	Hip	8	1.56 $\pm$ 0.08	1.38 $\pm$ 0.12	1.45	0.190

Table 4B. Effects of the rapid cooling regime on wood frog (*Rana sylvatica*) kinematics. Data are presented as means $\pm$ the standard error of the mean (SEM). Significant results are shown in **bold**. Angles are reported in radians.

Variable		<i>N</i>	Before	After	<i>t</i>	<i>p</i>
			Mean $\pm$ SEM	Mean $\pm$ SEM		
Jumping	Distance (mm)	12	237.62 $\pm$ 31.93	183.94 $\pm$ 28.15	1.56	0.150
	Avg Velocity (cm sec ⁻¹)	12	86.52 $\pm$ 9.49	42.82 $\pm$ 7.61	4.57	<0.001
	Max Height (mm)	12	8.72 $\pm$ 3.65	10.81 $\pm$ 4.94	0.36	0.730
	Jump Loading Time (msec)	9	20.67 $\pm$ 4.67	34.67 $\pm$ 6.27	1.84	0.100
Swimming	Avg Velocity (cm sec ⁻¹)	10	26.33 $\pm$ 3.48	18.26 $\pm$ 2.53	1.63	0.140
	Kick Distance (mm)	10	60.36 $\pm$ 7.49	46.62 $\pm$ 7.05	1.01	0.340
	Kick Velocity (cm sec ⁻¹)	10	41.29 $\pm$ 5.63	24.45 $\pm$ 4.13	2.49	0.030
	Distance Recovery (mm)	10	46.07 $\pm$ 7.30	60.53 $\pm$ 9.48	0.89	0.400
	Velocity Recovery (cm sec ⁻¹)	10	18.58 $\pm$ 2.45	17.78 $\pm$ 2.33	0.22	0.830
	Max Instantaneous Velocity (cm sec ⁻¹)	10	53.32 $\pm$ 6.95	34.02 $\pm$ 5.36	2.28	0.040
Maximum Extension Angles	Foot	10	3.12 $\pm$ 0.03	3.18 $\pm$ 0.05	0.97	0.360
	Ankle	10	3.03 $\pm$ 0.03	2.91 $\pm$ 0.05	2.02	0.070
	Knee	10	2.55 $\pm$ 0.04	2.37 $\pm$ 0.06	2.04	0.070
	Hip	10	2.74 $\pm$ 0.06	2.66 $\pm$ 0.04	1.88	0.090
Maximum Flexion Angles	Foot	10	4.39 $\pm$ 0.18	4.69 $\pm$ 0.15	1.14	0.280
	Ankle	10	0.96 $\pm$ 0.10	1.14 $\pm$ 0.08	0.89	0.400
	Knee	10	0.32 $\pm$ 0.07	0.36 $\pm$ 0.07	0.22	0.830
	Hip	10	1.49 $\pm$ 0.06	1.63 $\pm$ 0.07	1.02	0.340

Table 5. Effects of freezing on wood frog (*Rana sylvatica*) kinematics. Data are pooled across the rapid and slow cooling regimes to assess the general effects of freezing on kinematics. Data are presented as means $\pm$ the standard error of the mean (SEM). Significant results are shown in **bold**.

	Variable	<i>N</i>	Before Mean $\pm$ SEM	After Mean $\pm$ SEM	<i>t</i>	<i>p</i>
Jumping	Distance (mm)	24	199.51 $\pm$ 21.55	177.05 $\pm$ 19.58	0.86	0.400
	Avg Velocity (cm sec ⁻¹)	24	71.28 $\pm$ 6.74	46.18 $\pm$ 5.55	2.95	0.007
	Max Height (mm)	24	6.50 $\pm$ 2.61	9.78 $\pm$ 3.73	0.73	0.470
	Jump Loading Time (msec)	21	22.19 $\pm$ 3.54	47.25 $\pm$ 6.78	3.31	0.004
Swimming	Avg Velocity (cm sec ⁻¹)	18	27.64 $\pm$ 2.51	17.90 $\pm$ 1.94	2.76	0.013
	Kick Distance (mm)	18	67.07 $\pm$ 6.28	44.62 $\pm$ 4.34	2.46	0.025
	Kick Velocity (cm sec ⁻¹)	18	42.81 $\pm$ 4.28	25.11 $\pm$ 3.11	3.30	0.004
	Distance Recovery (mm)	18	47.28 $\pm$ 4.94	54.26 $\pm$ 6.08	0.71	0.490
	Velocity Recovery (cm sec ⁻¹)	18	19.72 $\pm$ 1.71	16.00 $\pm$ 1.74	1.37	0.190
	Max Instantaneous Velocity (cm sec ⁻¹)	18	54.95 $\pm$ 5.06	34.06 $\pm$ 3.99	3.10	0.007

Table 6A. Effects of the slow cooling regime on wood frog (*Rana sylvatica*) stroke cycle and muscle activation timings. Data are presented as means ± angular variance in radians. Significant results are shown in **bold**. Data that were non-directional were excluded from the analysis.

Variable			<i>N</i>	Before	After	<i>F</i>	<i>p</i>
				Mean ± Ang Var	Mean ± Ang Var		
Maximum Angular Timings	Foot	Extension	9	-1.12 ± 0.48	-1.54 ± 0.25	23.67	<0.001
		Flexion	9	0.59 ± 0.37	0.93 ± 0.11	12.38	0.003
	Ankle	Extension	9	2.09 ± 0.14	2.46 ± 0.21	10.04	0.005
		Flexion	9	-0.21 ± 0.06	-0.45 ± 0.16	2.19	0.160
	Knee	Extension	9	1.94 ± 0.09	2.29 ± 0.10	5.49	0.030
		Flexion	9	-0.50 ± 0.15	-0.70 ± 0.11	1.76	0.200
	Hip	Extension	9	2.25 ± 0.37	2.36 ± 0.13	1.19	0.290
		Flexion	9	-0.52 ± 0.07	-0.67 ± 0.14	0.90	0.360
EMG Timings	Gastrocnemius	Start	4	-0.89 ± 0.14	Non-Directional	NA	NA
		End	4	0.12 ± 0.09	Non-Directional	NA	NA
	Semimembranosus	Start	6	Non-Directional	Non-Directional	NA	NA
		End	6	Non-Directional	Non-Directional	NA	NA

Table 6B. Effects of the rapid cooling regime on wood frog (*Rana sylvatica*) stroke cycle and muscle activation timings. Data are presented as means $\pm$ angular variance in radians. Significant results are shown in **bold**. Data that were non-directional were excluded from the analysis.

Variable			<i>N</i>	Before Mean $\pm$ Ang Var	After Mean $\pm$ Ang Var	<i>F</i>	<i>p</i>
Maximum Angular Timings	Foot	Extension	11	-1.30 $\pm$ 0.55	-1.31 $\pm$ 0.61	0.02	0.890
		Flexion	11	0.86 $\pm$ 0.46	1.04 $\pm$ 0.28	6.65	0.020
	Ankle	Extension	11	2.44 $\pm$ 0.38	2.29 $\pm$ 0.35	4.40	0.049
		Flexion	11	-0.28 $\pm$ 0.14	-0.19 $\pm$ 0.07	0.39	0.540
	Knee	Extension	11	2.31 $\pm$ 0.16	2.39 $\pm$ 0.16	0.62	0.440
		Flexion	11	-0.48 $\pm$ 0.24	-0.58 $\pm$ 0.17	1.01	0.330
	Hip	Extension	11	2.50 $\pm$ 0.25	2.29 $\pm$ 0.20	5.55	0.030
		Flexion	11	-0.39 $\pm$ 0.06	-0.56 $\pm$ 0.15	1.24	0.280
EMG Timings	Gastrocnemius	Start	3	-0.95 $\pm$ 0.22	-0.57 $\pm$ 0.07	0.52	0.500
		End	3	0.56 $\pm$ 0.27	0.89 $\pm$ 0.06	0.46	0.520
	Semimembranosus	Start	3	-0.94 $\pm$ 0.10	Non-Directional	NA	NA
		End	3	0.22 $\pm$ 0.10	Non-Directional	NA	NA

Figure 1. Schematic representation of a frog. Six anatomical landmarks were digitized in the ventral view to calculate animal movement. The expanded region in the dorsal view shows the position of the two muscles examined in this study. The *m. semimembranosus* is a hip extensor and knee flexor that originates on the ischium and inserts on the medial tibiofibula. The *m. gastrocnemius* originates posteriorly on the femoral condyles and inserts on the plantar aponeurosis posterior of the tibiotarsal joints acting as a foot flexor, and also flexes the leg at the knee joint. Single bipolar electromyography electrodes were implanted into each muscle to measure activation patterns during jumping and swimming.

Figure 2. Timing of kinematic variables for the right leg during swimming before (thick grey) and after (thin black) freeze. One complete stroke cycle is represented by 360 degrees; 0 degrees represents the start of extension and 180 degrees the start of flexion. A) Timing of leg extension and flexion before and after the slow cooling treatment. B) Timing of leg extension and flexion before and after the rapid cooling treatment. C) EMG muscle activation/deactivation timing differences before and after the rapid cooling treatment.

Significant differences before and after treatment are denoted with an asterisk.