

## RESEARCH ARTICLE

## Real-time measurement of metabolic rate during freezing and thawing of the wood frog, *Rana sylvatica*: implications for overwinter energy use

Brent J. Sinclair<sup>1,\*</sup>, Joseph R. Stinziano<sup>1</sup>, Caroline M. Williams<sup>1,†</sup>, Heath A. MacMillan<sup>1</sup>, Katie E. Marshall<sup>1</sup> and Kenneth B. Storey<sup>2</sup>

<sup>1</sup>Department of Biology, University of Western Ontario, London, ON N6A 5B7, Canada and <sup>2</sup>Institute of Biochemistry, Carleton University, Ottawa, ON K1S 5B6, Canada

\*Author for correspondence ([bsincla7@uwo.ca](mailto:bsincla7@uwo.ca))

<sup>†</sup>Present address: Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611, USA

## SUMMARY

Ectotherms overwintering in temperate ecosystems must survive low temperatures while conserving energy to fuel post-winter reproduction. Freeze-tolerant wood frogs, *Rana sylvatica*, have an active response to the initiation of ice formation that includes mobilising glucose from glycogen and circulating it around the body to act as a cryoprotectant. We used flow-through respirometry to measure CO<sub>2</sub> production ( $\dot{V}_{\text{CO}_2}$ ) in real time during cooling, freezing and thawing. CO<sub>2</sub> production increases sharply at three points during freeze–thaw: at +1°C during cooling prior to ice formation (total of  $104 \pm 17 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ event}^{-1}$ ), at the initiation of freezing ( $565 \pm 85 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ freezing event}^{-1}$ ) and after the frog has thawed ( $564 \pm 75 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ thawing event}^{-1}$ ). We interpret these increases in metabolic rate to represent the energetic costs of preparation for freezing, the response to freezing and the re-establishment of homeostasis and repair of damage after thawing, respectively. We assumed that frogs metabolise lipid when unfrozen and that carbohydrate fuels metabolism during cooling, freezing and thawing, and when frozen. We then used microclimate temperature data to predict overwinter energetics of wood frogs. Based on the freezing and melting points we measured, frogs in the field were predicted to experience as many as 23 freeze–thaw cycles in the winter of our microclimate recordings. Overwinter carbohydrate consumption appears to be driven by the frequency of freeze–thaw events, and changes in overwinter climate that affect the frequency of freeze–thaw will influence carbohydrate consumption, but changes that affect mean temperatures and the frequency of winter warm spells will modify lipid consumption.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/216/2/292/DC1>

Key words: energetics, freeze tolerance, respirometry.

Received 18 June 2012; Accepted 27 September 2012

## INTRODUCTION

Ectotherms that overwinter without feeding are wholly dependent on their body energy reserves to fuel winter metabolism, as well as development, activity and reproduction in the spring. For example, reproductive output of female goldenrod gall flies is determined by lipid reserves remaining in the spring (Irwin and Lee, 2000). Because metabolism of ectotherms (and many hibernating mammals) is determined by the ambient temperature, overwinter energetics can mediate the relationship between environmental conditions and the persistence and performance of populations, and therefore the geographic distribution of animals (Humphries et al., 2002). Overwinter energy use is determined not only by direct temperature–metabolic rate relationships, but also by physiological thresholds, which can also influence energy use; for example, lower critical temperature signals have been shown to increase energetic costs of thermoregulation in mammals (Humphries et al., 2002), and metabolic suppression associated with ice formation reduces overwinter energy consumption in insects (Marshall and Sinclair, 2012). Thus, identifying these thresholds, and the magnitude of their effects, is an important component of understanding the energetics of overwintering.

Some frogs, turtles and lizards are freeze tolerant, surviving internal ice formation as part of their overwintering strategy (Storey and Storey, 1992). The freezing process is usually initiated by contact with external ice; however, ice-nucleating agents have been found in the skin of the brown tree frog, *Litoria ewingii*, and in the blood and gut of the wood frog, *Rana sylvatica* (Wolanczyk et al., 1990; Lee and Costanzo, 1998; Rexer-Huber et al., 2011). In *R. sylvatica*, the best-studied freeze-tolerant vertebrate, ice formation initiates a signalling cascade that leads to the production of a variety of stress-related proteins and the mobilisation of glycogen from liver reserves. Glycogen is cleaved into glucose, which acts as a cryoprotectant (Layne and Lee, 1995; Storey and Storey, 1996; Storey, 2004; Storey, 2008). Once freezing is initiated, maximal ice formation takes 24 h or more in *R. sylvatica* (Lee et al., 1992), during which cardiac function can continue for more than 12 h (Costanzo et al., 1993). Although ventilation ceases as ice content in the body increases, frogs may also respire cutaneously until freezing ceases circulation to the skin. Unfrozen overwintering frogs probably primarily catabolise lipids [cf. frogs overwintering in water (Tattersall and Ultsch, 2008)], but once the frog is completely frozen, metabolism is largely anaerobic, and is mainly fuelled by

carbohydrates (Storey and Storey, 1986). Cardiovascular function of *R. sylvatica* is re-established within 1 h of thawing, with recovery of breathing and hind leg reflexes taking longer (Layne and First, 1991). During thawing, glucose is cleared either directly from the blood or after reabsorption from the bladder, and converted back to glycogen in the liver (Storey and Storey, 1986; Costanzo et al., 1997).

The production, distribution and re-sequestration of carbohydrate cryoprotectants have energetic costs. The metabolic rate of frogs is higher after thawing (Layne and Kefauver, 1997; Layne, 2000; Voituron et al., 2009), suggesting that the energetic cost of freezing and thawing likely includes additional costs beyond the simple effect of temperature on metabolic rate, such as repair of accumulated damage, re-establishment of osmotic and ionic gradients, and re-synthesis of damaged proteins. Metabolic rate in frozen frogs may be suppressed due to high concentrations of solutes, reduced cellular volume and accumulated urea (Muir et al., 2008). To date, measurements of metabolism associated with low temperature in freeze-tolerant amphibians and reptiles have been conducted with closed-flow systems and/or at static temperatures (Voituron et al., 2002a; Voituron et al., 2009), which means that the direct metabolic costs of the freezing and thawing processes are unknown.

Real-time measurement of metabolic rate with temperature (or other) changes is possible using both direct calorimetry (Hansen et al., 2006) and respirometry (Lighton and Turner, 2004). Sinclair et al. (Sinclair et al., 2004) measured metabolic rate in a freeze-tolerant tenebrionid caterpillar, concluding that freezing and thawing in this insect did not have an energetic cost. The release of a large amount of CO<sub>2</sub> associated with the onset of freezing was hypothesised to be caused by the compression of the tracheal system with ice expansion in the body, rather than by an increase in metabolism or change in CO<sub>2</sub>-buffering capacity of the haemolymph.

Voituron et al. (Voituron et al., 2002b) predicted that freeze tolerance should be favoured over freeze avoidance as an energy conservation strategy; however, an important assumption of this model is that any direct costs of a freezing event are offset by energy savings when frozen. Thus, a model of overwinter energy costs would need to include not only the temperature effects on metabolic rate, but also the energetic costs of freezing and thawing, the degree to which metabolic rate is suppressed when frozen, and some consideration of the substrates used to fuel metabolism during winter. Total depletion of carbohydrate reserves will prevent the production of glucose cryoprotectants, leading to death if the frog freezes. Depletion of lipid energy reserves also has potential fitness consequences, as *R. sylvatica* does not feed before breeding in the early spring (K.B.S., unpublished). Thus, the energy reserves remaining at the end of winter are all that is available to fuel energetically costly activities associated with reproduction, including locomotion, calling, amplexus and egg production (Irwin et al., 2003; McLister, 2003). A winter energy budget has not previously been developed for any overwintering, freeze-tolerant vertebrate, but such a model would allow a test of the assumptions of Voituron et al. (Voituron et al., 2002b).

Here we use open-flow respirometry to measure CO<sub>2</sub> release (as a proxy to metabolic rate) in *R. sylvatica* during a short freeze–thaw cycle. We determine the cost of freezing and thawing, and use these values to parameterise a numerical simulation of overwinter energy use and freeze–thaw based on microclimate temperatures from two locations over a single winter. This allows us to partition the utilisation of carbohydrate and lipid energy reserves, and to determine the relative importance of freezing tolerance to the overwinter energetics of this species.

## MATERIALS AND METHODS

### Microclimate recordings

Microclimate temperatures were recorded hourly with iButton DS1922L Thermochron data loggers (Maxim-Dallas Semiconductor, Sunnyvale, CA, USA) placed on the ground beneath 2–5 cm of leaf litter and attached to a steel peg with a length of non-conductive string. The data loggers were placed in a woodlot near Ottawa dominated by black walnut (*Juglans nigra*), ash (*Fraxinus* spp.) and cherry (*Prunus* spp.). Temperatures were recorded at two locations ~30 m apart in the 2008–2009 winter. The data logger site is within 60 km of the site where frogs were collected, and wood frogs overwinter underneath leaf litter in forests such as this (Regosin et al., 2003; Baldwin et al., 2006) and have been present in all such woodlots that have been investigated (K.B.S., personal observation). For the purposes of numerical simulation (below), we used data from 1 November to 16 April to represent winter, as during our recordings, this period encompassed all instances of sub-zero microclimate temperatures. The soil data loggers were supplemented with an unshielded data logger at 2 m on the north-facing side of a nearby tree ('canopy temperature'), which was expected to approximate meteorological air temperature observations (with the exception that it is exposed to some solar radiation; see supplementary material Fig. S1 for a detailed comparison).

### Animal collection and care

Male *Rana sylvatica* LeConte 1825 were collected from woodlands near Ottawa. Autumn-collected frogs were collected in the second week of October 2010, held on crushed ice and transferred to Carleton University. Spring-collected frogs were collected at breeding ponds on 10–11 April 2011, kept in coolers on snow and transferred to Carleton University. All frogs were given an initial wash in a tetracycline bath, then placed in plastic boxes with a bed of damp sphagnum moss and held in an incubator at +3°C. Frogs were transferred to the University of Western Ontario in containers cooled with ice packs in mid-January (autumn-collected) or mid-April (spring-collected) 2011. Prior to being used in experiments, frogs were held in groups of 10 with moist sphagnum moss in 2.6 l plastic containers in the dark at +4±1°C. After experiments, frogs were held individually on a moist paper towel in 720 ml plastic containers at +4±1°C. Laboratory experiments were conducted under University of Western Ontario animal care guidelines, protocol AUP-2010-016.

### Respirometry

Respirometry was conducted in an 85 cm<sup>3</sup> acrylic chamber sealed at each end with an aluminium plug. A channel was milled into the underside of the chamber to reduce its thickness and allow freezing of the frog to be monitored with three 36 AWG Type-T thermocouples (Omega, Laval, QC, Canada) applied to the outside of the bottom of the chamber. The thermocouple that gave the clearest signal of freezing (and was therefore positioned closest to the frog) was used in analyses of each data set. A fourth, reference thermocouple was placed on top of the chamber; this reference thermocouple allowed us to detect deviations between the trajectory of bath and chamber temperatures and thus identify freezing and melting events. Thermocouples were connected to a TC2000 and recorded via a UI2 analog–digital interface (both Sable Systems International, Las Vegas, NV, USA). Activity was monitored with a Sable Systems AD-1 infrared activity detector. The chamber containing the frog, the reference thermocouple and an identical reference chamber (used as a baseline) were then placed into a plastic bag and submerged in the bath of a refrigerated circulator (Proline

RP3530, Lauda, Wurzburg, Germany) containing 50:50 ethylene glycol:water (v/v).

Dry, CO<sub>2</sub>-free air was directed through either the baseline chamber or the chamber containing the frog by a BL-2 baseline unit (Sable Systems International) at ~46 ml min<sup>-1</sup> [controlled by a mass flow valve (Sierra Instruments, Monterey, CA, USA) and measured by an SS3 sub-sampler (Sable Systems International)]. The air was pre-cooled before entering the baseline chamber by being passed through a 172 cm×6.2 mm copper coil tube submerged in the bath fluid. Water vapour was scrubbed post-animal with magnesium perchlorate, and CO<sub>2</sub> emission was then determined with a CA-10 CO<sub>2</sub> analyser (Sable Systems International). All instruments were interfaced to a computer using a UI2 AD interface (Sable Systems International), and data were acquired at 1 s intervals using Expedata software (Sable Systems International).

Frogs were weighed (Mettler PE 300, Columbus, OH, USA) and a silver iodide paste (1:4 AgI:water, v:v) was applied to the bottom of both hind feet to inoculate freezing before they were placed in the respirometry chamber at +4°C for 130 min (autumn-collected frogs) or 190 min (spring-collected frogs), which included a 20 min baseline recording. They were then cooled at 0.1°C min<sup>-1</sup> to 0°C, and from 0 to -2.4°C at 0.05°C min<sup>-1</sup>. Frogs were held at -2.4±0.1°C for 12 h, and warmed to +4°C at 0.5°C min<sup>-1</sup>, after which they were held at +4°C for 7.5 h (spring-collected frogs) or 6.5 h (autumn-collected frogs), followed by another baseline recording.

#### Data analysis

Freezing was identified by a clear exotherm, and melting by a departure of the temperature measured proximal to the frog from the reference temperature. The end of the thaw period was identified

by an increase in temperature after the conclusion of the melt period (Fig. 1). We calculated the rate of CO<sub>2</sub> release at periods when temperature was constant (before cooling, when frozen and after recovery from freezing), and integrated beneath peaks of CO<sub>2</sub> release prior to and during the freezing process, and during the period from the start of rewarming to the end of the melt endotherm. Thermal sensitivity coefficients ( $Q_{10}$ ) were calculated during cooling (prior to freezing) and during rewarming (prior to endotherm inflection) using the equation:

$$Q_{10} = 10^{10 \times \left( \frac{d \log_{10} \dot{V}_{CO_2}}{dT} \right)}, \quad (1)$$

where  $d \log_{10} \dot{V}_{CO_2} / dT$  is the slope of the linear regression of log<sub>10</sub> CO<sub>2</sub> production ( $\dot{V}_{CO_2}$ ) and temperature ( $T$ ). Several frogs were excluded from analyses because their body position blocked airflow, obscuring the ventilation patterns and damping the CO<sub>2</sub> trace. The equilibration period of 130 min used for autumn-collected frogs was too short to allow estimation of CO<sub>2</sub> emission during that period, so we excluded them from the calculation of pre-cooling CO<sub>2</sub> emission and  $Q_{10}$  during cooling.

All statistical comparisons were made of CO<sub>2</sub> emission, and means ± s.e.m. are reported throughout. All statistical analyses were conducted in R v.2.13 (R Development Core Team, 2010). CO<sub>2</sub> and  $\dot{V}_{CO_2}$  were ln-transformed and tested for homoscedasticity using Levene's test for homogeneity of variance in the lawstat package in R (Hui et al., 2008). Comparisons between means were conducted using ANCOVA with initial body mass as a covariate, and temperature of crystallisation ( $T_c$ ) and body mass were compared among spring- and autumn-collected frogs using Welch's *t*-tests.

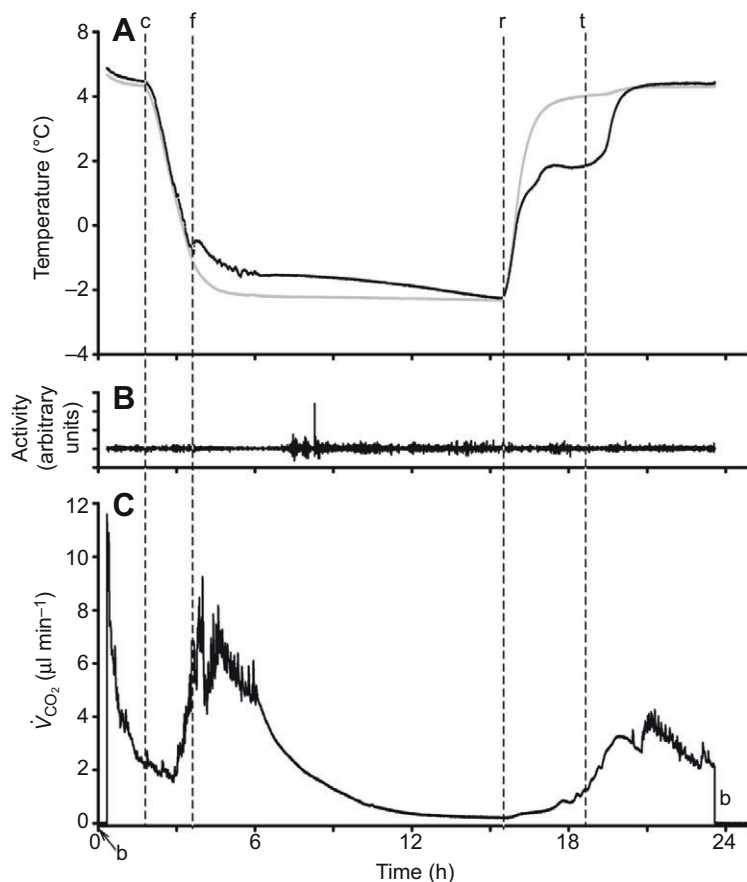


Fig. 1. Sample trace from a wood frog, *Rana sylvatica*, during freezing and thawing in the respirometer. (A) Reference temperature (grey) and frog temperature (black). (B) Activity (recorded from an infrared activity detector). (C) CO<sub>2</sub> production ( $\dot{V}_{CO_2}$ ). b, baseline recordings. Vertical dashed lines indicate: c, beginning of cooling; f, beginning of freezing exotherm; r, beginning of rewarming; and t, increase of temperature, indicating end of thawing.

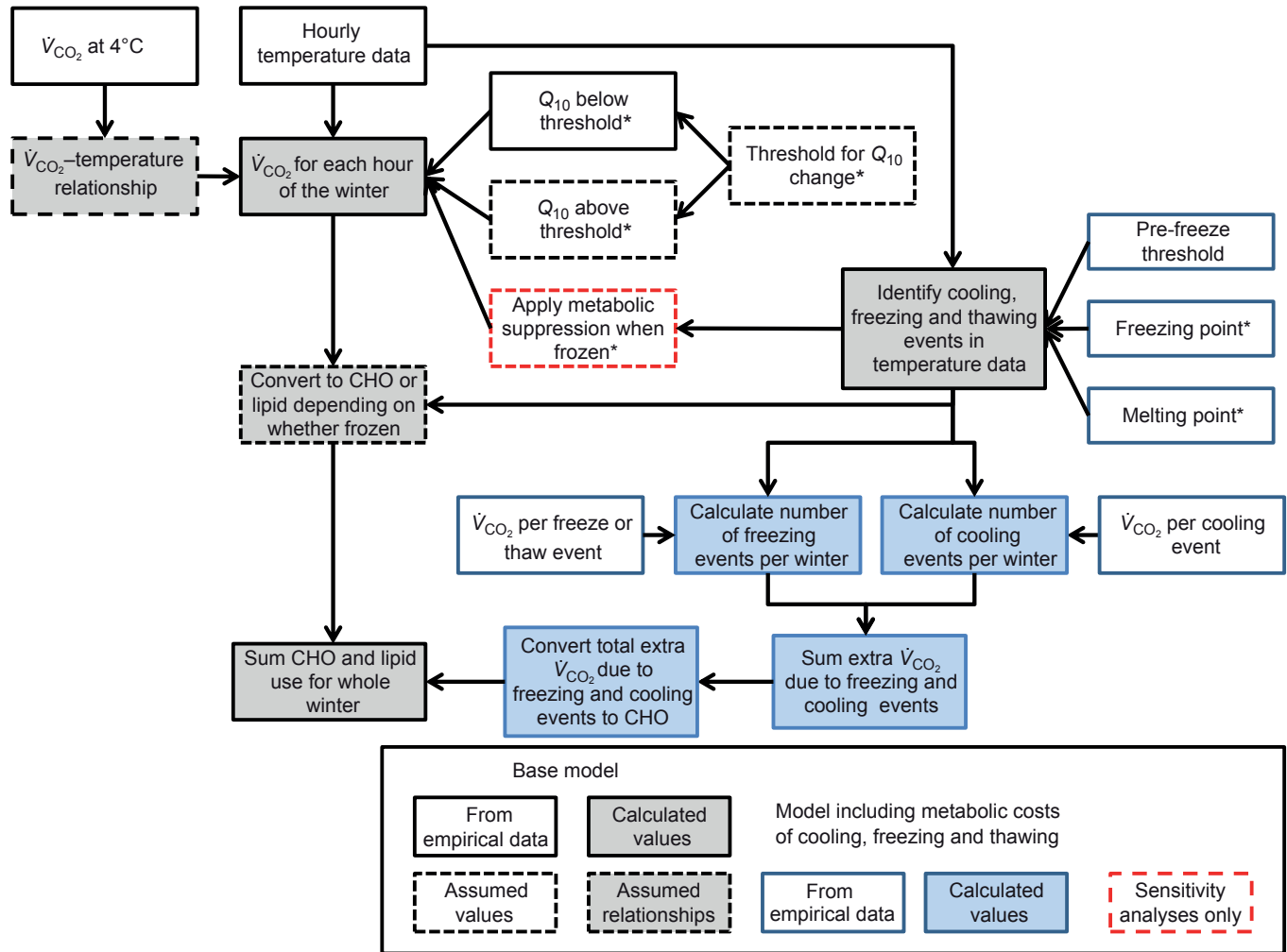


Fig. 2. Schematic of model of energy use for overwintering frogs, based upon the general model of Marshall and Sinclair (Marshall and Sinclair, 2012). Boxes with black borders are the base model. Boxes with blue borders indicate a model that includes metabolic costs of cooling, freezing and thawing. The box with a red border indicates a term present only in some sensitivity analyses. Solid borders indicate values derived from empirical data (present study); dashed borders indicate assumptions of the model. Shaded boxes indicate calculated terms. Asterisks indicate terms that were modified in sensitivity analyses.

### Numerical simulation of overwintering energetics

We predicted overwinter energy consumption from microclimate data using a combination of empirical data and assumptions supported by the literature (Fig. 2). We used the  $CO_2$  emission traces to derive a temperature- $\dot{V}_{CO_2}$  relationship, and also identified three periods during cooling, freezing and thawing when  $\dot{V}_{CO_2}$  was substantially in excess of that expected from this relationship. These were: (1) an increase in  $\dot{V}_{CO_2}$  during cooling, but before the initiation of ice formation (which we henceforth call the ‘cooling threshold\*’); (2) an increase in  $\dot{V}_{CO_2}$  upon freezing; and (3) an increase in  $\dot{V}_{CO_2}$  during thawing (Fig. 1). We assumed that lipid was the main fuel source when the frogs were unfrozen [as is the case for all dormant frogs that have been studied (Fitzpatrick, 1976; Long, 1987; Tattersall and Ultsch, 2008)], but that metabolism during freezing, thawing and while frozen was fuelled by carbohydrate (Storey and Storey, 1986). We then used this simulation to predict  $CO_2$  production from microclimate data (see Marshall and Sinclair, 2012) from the microhabitat temperature recordings, and calculated expected total overwinter carbohydrate and lipid consumption (Fig. 2). We varied each of the parameters

in our model in turn to determine the sensitivity of the conclusions to our assumptions.

First, we used the mean  $CO_2$  production at +4°C ( $0.41 \mu l CO_2 \text{ min}^{-1} \text{ g}^{-1}$ ) and the mean  $Q_{10}$  of  $CO_2$  emission during cooling but before  $\dot{V}_{CO_2}$  increased prior to freezing (effectively during cooling from +3.5 to +1.8°C) to generate a simulated temperature- $\dot{V}_{CO_2}$  ( $\text{ml h}^{-1} \text{ g}^{-1}$ ) relationship to provide parameters  $a$  and  $b$  for the equation:

$$\dot{V}_{CO_2} = a10^{Tb}. \quad (2)$$

Because the  $Q_{10}$  estimated during cooling (7.97) was unusually large, we repeated this to obtain coefficients assuming a  $Q_{10}$  of 2.0, which is at the low end expected for biochemical reactions (Hochachka and Somero, 2002). For our modelling, we expected (from most other biological systems) that thermal sensitivity would be less than 8.0 at higher temperatures, so we assumed a  $Q_{10}$  of 2.0 at temperatures higher than an arbitrary threshold of +4°C, and a  $Q_{10}$  of 7.97 at temperatures of +4°C and below. We explored the impact of these assumptions in our sensitivity analyses. From our empirical data, we obtained the average  $CO_2$

production at the cooling threshold (prior to ice formation) of  $104.6 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ event}^{-1}$  at an average threshold temperature of  $+1.03^\circ\text{C}$ . From our empirical data we estimated the additional  $\text{CO}_2$  production associated with the freezing event to be  $565 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ freezing event}^{-1}$  and an additional  $564 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ freezing event}^{-1}$  associated with thawing. We assumed that any drop in temperature below the mean  $T_c$  would result in the full energetic cost of freezing, and the energetic cost of thawing was applied only when the temperature increased above the mean temperature where the melt endotherm ended ( $-0.16^\circ\text{C}$ ).

Previous studies have suggested that metabolic rate is depressed beyond direct temperature effects when frozen (Voituron et al., 2009; Marshall and Sinclair, 2012; Zhang and Storey, 2012), but using the  $Q_{10}$  of 7.97, we estimated a  $\text{CO}_2$  production of frogs frozen at  $-3.5^\circ\text{C}$  ( $9.588 \times 10^{-5} \text{ ml CO}_2 \text{ min}^{-1} \text{ g}^{-1}$ ) that was the same order of magnitude as that observed empirically ( $2.450 \times 10^{-5} \text{ ml CO}_2 \text{ min}^{-1} \text{ g}^{-1}$ ), so we did not employ any freeze-related depression of metabolism in our main simulation, likely leading to overestimates of energy use while frozen. However, we did explore the consequences of this assumption in our sensitivity analyses. Previous studies have indicated that while frozen, *R. sylvatica* is dependent on carbohydrate metabolism (Storey and Storey, 1986), and we assumed that, when unfrozen, lipid stores provide the main fuel source (Fitzpatrick, 1976; Long, 1987; Tattersall and Ultsch, 2008). We assumed that carbohydrates were the fuel source during bursts of  $\text{CO}_2$  production in cooling, freezing and thawing, and that carbohydrates provided all metabolic fuel when the frogs were frozen. To calculate total lipid consumption, we assumed a respiratory quotient (RQ) of 0.7 and that 2 ml of oxygen is consumed per milligram of lipid oxidized. Total carbohydrate consumption was calculated assuming an RQ of 1.0 and that 0.84 ml of oxygen is consumed per milligram of carbohydrate oxidized. Total energy use was calculated by converting substrate mass to kJ, assuming  $17 \text{ kJ g}^{-1}$  carbohydrate and  $39 \text{ kJ g}^{-1}$  lipid.

We calculated  $\text{CO}_2$  production for each hour of temperature recording, assuming that the temperature remained the same for that hour (see also Marshall and Sinclair, 2012; Williams et al., 2012), and that a frog's body temperature is equilibrated with that of its surrounding microhabitat (this comprises the base model). We then identified events where the temperature dropped below the 'cooling threshold', and freeze-thaw events using an approach based on that of Sinclair (Sinclair, 2001), but implemented in R (code available from the authors upon request) (see also Marshall and Sinclair, 2012), and applied the metabolic costs of those events as described above.

Frogs were assumed to freeze any time the temperature dropped below the freezing threshold, and to thaw any time the temperature increased above the melting point, and we scaled most of our predictions for a 7.1 g frog (the mean mass of the frogs we used).

We explored the sensitivity of the results of the model to our assumptions and estimated parameters. First, we explored the impact of our assumptions about  $Q_{10}$ , and also our assumptions about a change in  $Q_{10}$  at threshold temperatures. We used a  $Q_{10}$  of 4.0 (Voituron et al., 2009); a change to a  $Q_{10}$  of 1.5 above  $+4^\circ\text{C}$ ; a threshold for  $Q_{10}$  change of 0 and  $8^\circ\text{C}$ ; and a  $Q_{10}$  of 2.0 or 7.97 without a threshold change. Second, we modified the costs of freezing and thawing, and made the freezing and melting points more conservative (shift of  $T_c$  to  $-0.8^\circ\text{C}$ , and melting point to  $-0.6^\circ\text{C}$ ). Finally, we introduced a depression of metabolic rate when frozen to 50, 5 or 0.5% of the value calculated from our equation.

## RESULTS

### Metabolism during cooling, freezing and thawing

Body mass was allometrically related to  $\dot{V}_{\text{CO}_2}$  at  $+4^\circ\text{C}$  with an exponent of  $0.744 \pm 0.305$  ( $F_{1,10} = 5.97$ ,  $P = 0.035$ ), and was retained as a covariate in subsequent analyses.  $\text{CO}_2$  emission by frogs during cooling, freezing and thawing was qualitatively similar for all individuals ( $N = 12$ ), and an example trace is shown in Fig. 1C. Upon cooling from  $+4^\circ\text{C}$ ,  $\text{CO}_2$  production decreased ( $Q_{10} = 7.97 \pm 0.39$ ) until a threshold of  $1.03 \pm 0.1^\circ\text{C}$  was reached, when  $\text{CO}_2$  production increased markedly. This  $\text{CO}_2$  increase was not associated with increased activity (apart from ventilation), and we call it the 'pre-freeze increase' in  $\text{CO}_2$  production. This pre-freeze increase released a mean of  $104 \pm 17 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ event}^{-1}$ , and the maximum  $\dot{V}_{\text{CO}_2}$  ( $0.73 \pm 0.049 \mu\text{l CO}_2 \text{ min}^{-1} \text{ g}^{-1}$ ) was always lower than subsequent  $\dot{V}_{\text{CO}_2}$  maxima during freezing. Freezing was readily detected by the thermocouples, and was accompanied by a further sharp increase in  $\dot{V}_{\text{CO}_2}$ , to a mean  $\dot{V}_{\text{CO}_2}$  maximum of  $1.11 \pm 0.11 \mu\text{l CO}_2 \text{ min}^{-1} \text{ g}^{-1}$ . Accounting for the projected baseline decline in  $\text{CO}_2$  production associated with decreasing temperature, the increase in  $\text{CO}_2$  production with freezing amounts to  $565 \pm 85 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ freezing event}^{-1}$ . Ventilation was identifiable as regular inflections of the  $\text{CO}_2$  trace (Fig. 1). Ventilation ceased  $76.9 \pm 8.8 \text{ min}$  (range: 24–150 min) after the initiation of the exotherm (Fig. 1), but  $\text{CO}_2$  emission remained elevated for  $465 \pm 20 \text{ min}$  after the start of freezing, and some activity (presumably movement of the frog stimulated by expansion of ice) occurred after the cessation of breathing in some cases. While frozen,  $\dot{V}_{\text{CO}_2}$  remained stable at  $0.174 \pm 0.020 \mu\text{l min}^{-1} \text{ frog}^{-1}$  (Table 1).

Table 1. Respirometry parameters measured in *Rana sylvatica* during cooling, freezing and thawing

	Overall mean	Autumn	Spring	Statistic	P
N	12	5	7		
Body mass (g)	$7.09 \pm 0.50$	$6.93 \pm 0.94$	$7.26 \pm 0.43$	$t_{10} = 0.17$	0.865
Mass lost (g)	$0.60 \pm 0.08$	$0.43 \pm 0.05$	$0.77 \pm 0.11$	$F_{1,10} = 5.34$	0.044
Temperature of crystallisation ( $^\circ\text{C}$ )	$-1.4 \pm 0.1$	$-1.6 \pm 0.1$	$-1.2 \pm 0.1$	$t_{10} = 3.71$	<0.01
Pre-freeze increase threshold ( $^\circ\text{C}$ )	$1.03 \pm 0.11$	$1.30 \pm 0.15$	$0.87 \pm 0.15$	$t_{10} = 2.25$	0.052
Melting point ( $^\circ\text{C}$ )	$-0.16 \pm 0.01$	$-0.16 \pm 0.01$	$-0.16 \pm 0.01$	$t_{10} = 0.36$	0.724
$Q_{10}$ during rewarming	$16.61 \pm 5.68$	$19.64 \pm 9.17$	$12.38 \pm 5.37$	$t_{10} = 0.68$	0.512
$\dot{V}_{\text{CO}_2}$ precooling ( $\mu\text{l min}^{-1} \text{ g}^{-1}$ )	$0.410 \pm 0.036$	$0.426 \pm 0.028$	$0.399 \pm 0.052$	$F_{1,10} = 0.43$	0.525
$\dot{V}_{\text{CO}_2}$ while frozen ( $\mu\text{l min}^{-1} \text{ g}^{-1}$ )	$0.0245 \pm 0.0025$	$0.0262 \pm 0.0051$	$0.0233 \pm 0.0027$	$F_{1,10} = 0.06$	0.818
$\dot{V}_{\text{CO}_2}$ after thawing ( $\mu\text{l min}^{-1} \text{ g}^{-1}$ )	$0.406 \pm 0.024$	$0.409 \pm 0.038$	$0.405 \pm 0.034$	$F_{1,10} < 0.01$	0.999

Autumn frogs were collected pre-winter, while frogs collected in the spring had presumably experienced a winter of freeze-thaw cycles. Statistics compare pre- and post-winter values, and although mass-specific values are presented for some parameters, analyses were performed as ANCOVAs. The pre-freeze increase was an increase in  $\dot{V}_{\text{CO}_2}$  during cooling, but prior to the initiation of ice formation.

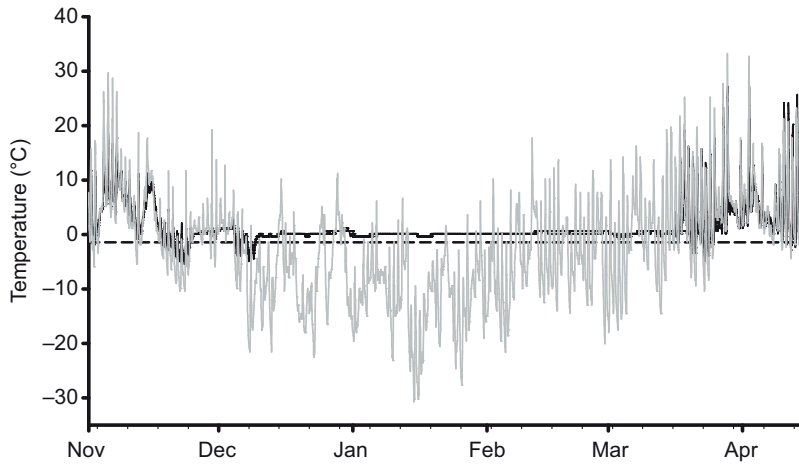


Fig. 3. Microclimate temperatures representative of *Rana sylvatica* winter habitat measured by iButton data loggers over the winter of 2008–2009 (1 November–16 April;  $N=2$  data loggers shown). Air temperatures (recorded from an unshielded data logger in the canopy) are included for comparison. Black=ground temperature (no distinction made between the two locations); grey=air temperature. The dashed horizontal line shows the frog freezing temperature of  $-1.4^{\circ}\text{C}$  used in our base models.

Upon rewarming,  $\dot{V}_{\text{CO}_2}$  increased rapidly (Fig. 1). The beginning of thawing was identifiable by a deflection of the frog temperature, and began at  $-0.16 \pm 0.01^{\circ}\text{C}$ . Thawing took  $150.4 \pm 8.2$  min to complete, and ventilation resumed  $93.6 \pm 12.3$  min after the completion of thawing. The total  $\text{CO}_2$  production associated with thawing from the beginning of rewarming to the resumption of ventilation was  $564 \pm 75 \mu\text{l CO}_2 \text{ frog}^{-1} \text{ freezing event}^{-1}$ .

The temperature of crystallisation was lower in autumn-collected frogs than in frogs collected in spring (Table 1). There was no significant difference between body mass of frogs collected in autumn and spring (Table 1); however, the autumn-collected frogs lost less mass during the respirometry run (Table 1), probably because the respirometry runs were approximately 5 h shorter for the autumn- versus spring-collected frogs, resulting in less evaporative water loss.

#### Microclimate temperatures

Microclimate temperatures for the 2008–2009 winter (1 November–16 April) from the Ottawa region are shown in Fig. 3 and summarised in Table 2. The buffering effect of leaf litter and snow reduced the amplitude of microclimate temperature fluctuations compared with air temperatures, but the leaf litter temperatures were also increased on clear days due to solar radiation (Fig. 3; supplementary material Fig. S1). Although the mean air temperature was only slightly lower than the means recorded in the leaf litter (Table 2), the minimum temperature recorded in air ( $-30.7^{\circ}\text{C}$ ) was much lower than the minimum temperature measured in the leaf litter ( $-5.4^{\circ}\text{C}$  at location 2). Snow cover meant that most of the freeze–thaw cycles in the leaf litter were confined to the spring and autumn (Fig. 3). Microclimate data show mean predicted lengths of freeze–thaw cycles (14–17 h) similar to the freezing exposures

used in the present study. However, the maximum length (76 h) was substantially longer than that used in our experiments.

#### Numerical simulation

Our model and the microclimate recordings predict that, in the leaf litter, frogs would have experienced as many as 23 freeze–thaw cycles in the winter we observed, and would have spent 6–8% of the winter frozen (Table 2). The longest single period a frog was predicted to be frozen lasted 76 h (Table 2). Comparison of the leaf litter and air temperatures indicate that the microclimate is considerably buffered compared with air temperatures (Table 2). Calculation of predicted frog freeze–thaw events from air rather than microclimate temperatures suggests a higher number of freeze–thaw cycles, longer periods spent frozen and an increased length of freeze–thaw cycles in air compared with leaf litter.

Using our base model (assuming no cost to freezing and thawing), we predicted that frogs would use  $1.5\text{--}1.6 \text{ kJ g}^{-1}$  over the winter (Fig. 4). The total predicted energy use was increased by  $<10\%$  when the cost of freezing and thawing and the ‘pre-freeze increase’ of metabolism were included (Fig. 4). Most of the energy use was during the spring and autumn, when a lack of snow cover resulted in more extreme temperature fluctuations, including high temperatures, which result in disproportionately high energy use due to Jensen’s inequality (Ruel and Ayres, 1999; Williams et al., 2012) (Figs 4, 5). Using our assumptions about fuel sources, a 7.1 g frog would be expected to consume 0.28 to 0.37 g lipid and 0.05 to 0.12 g carbohydrate per winter (Table 3).

Sensitivity analyses (Table 4) identify several assumptions of our model that have an impact on our estimates of the number of freeze–thaw cycles and overwinter energy use. Increasing the  $T_c$  from  $-1.36$  to  $-0.8^{\circ}\text{C}$  increased the number of freeze–thaw cycles

Table 2. Summary of overwinter microclimate temperatures from a woodlot near Ottawa, in the winter (1 November–16 April) of 2008–2009

	Location 1	Location 2	Air
Mean ( $\pm$ s.d.) temperature ( $^{\circ}\text{C}$ )	$1.2 \pm 3.1$	$1.5 \pm 3.9$	$-2.6 \pm 9.4$
Maximum temperature ( $^{\circ}\text{C}$ )	21.2	27.7	33.2
Minimum temperature ( $^{\circ}\text{C}$ )	-4.9	-5.4	-30.7
Number of frog freeze–thaw cycles	16	23	85
Total time spent frozen (h)	270	312	2202
Time spent frozen (% of winter)	6.8	7.9	56
Range of freeze–thaw cycle lengths (h)	2–76	2–63	1–208
Mean ( $\pm$ s.d.) length of freeze–thaw cycles (h)	$17 \pm 19$	$14 \pm 12$	$26 \pm 37$

Locations 1 and 2 indicate different data logger locations, ‘Air’ indicates a data logger placed in the canopy 2 m above the ground. Freeze–thaw cycles are for frogs, assuming a temperature of crystallisation of  $-1.36$  and a melting point of  $-0.16^{\circ}\text{C}$  (see Materials and methods for details).

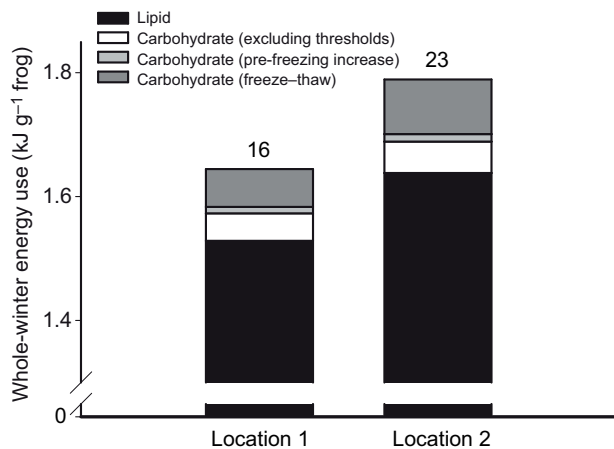


Fig. 4. Breakdown of predicted energy consumption by a 7.1 g *Rana sylvatica* for two locations over the 2008–2009 winter. It is assumed that metabolism outside of freeze–thaw periods is fuelled by lipid, but that when frogs are frozen they utilise carbohydrate. The pre-freezing increase accounts for an observed increase in metabolism during cooling, and the freeze–thaw value accounts for the observed increase in metabolic rate associated with freezing and thawing processes. See Results for further details. Numbers above bars indicate the estimated number of freeze–thaw cycles for a location.

and time spent frozen by ~20%, which had a comparable impact on predicted winter carbohydrate consumption. Lowering the melting point from  $-0.16$  to  $-0.6^{\circ}\text{C}$  had no impact on the predicted number of freeze–thaw cycles, although it did reduce the time spent frozen by 7%, which therefore slightly increased lipid consumption and reduced carbohydrate consumption. When  $T_c$  was increased to  $-0.8^{\circ}\text{C}$  and melting point decreased to  $-0.6^{\circ}\text{C}$ , the number of freeze–thaw cycles increased by 39%, but the time spent frozen decreased by only 7%. Consequently, this scenario led to <1% lipid savings, but a 26% increase in predicted carbohydrate consumption relative to the base model.

We observed a high  $Q_{10}$  (7.97) of  $\dot{V}_{\text{CO}_2}$  during cooling from  $+4^{\circ}\text{C}$ , so our base model assumed a shift to a lower thermal sensitivity ( $Q_{10}=2.0$ ) above a specific threshold ( $+4^{\circ}\text{C}$ ). Decreasing the threshold at which  $Q_{10}$  increases from  $+4$  to  $0^{\circ}\text{C}$  increased the predicted

overwinter lipid consumption by 31%, but raising that threshold to  $+8^{\circ}\text{C}$  increased the predicted lipid consumption by only 4%, so the high  $Q_{10}$  we observed results mainly in a suppression of metabolic rate at these temperatures. Neither change in  $Q_{10}$  threshold affected the predicted carbohydrate consumption (Table 4). Changing  $Q_{10}$  to a constant value of 2.0 increased both lipid and carbohydrate consumption by ~40%, and using a  $Q_{10}$  of 7.97 throughout increased our prediction of lipid consumption by 137%. Assuming that metabolic rate is depressed when the frog is frozen decreases predicted carbohydrate consumption by 17–33%, the value increasing as the magnitude of the depression was increased (Table 4).

## DISCUSSION

Previous studies of metabolism in freeze-tolerant vertebrates (e.g. Voituron et al., 2002a; Voituron et al., 2009) have employed a closed-system approach that does not allow the patterns of metabolic changes to be observed, nor does it allow the direct estimation of the metabolic costs of the freezing and thawing processes. Here we show that  $\text{CO}_2$  production by *R. sylvatica* during freezing and thawing is dynamic. In particular, we noted an increase in  $\text{CO}_2$  production during cooling before the onset of freezing, an additional increase in  $\text{CO}_2$  production coincident with ice formation, and an ‘overshoot’ of  $\text{CO}_2$  production following thawing.

The increases in  $\text{CO}_2$  production associated with cooling, freezing and thawing were not associated with increased movement (Fig. 1). Furthermore, although electrical noise prevented us from making quantitative measurements, oxygen consumption increased in tandem with  $\dot{V}_{\text{CO}_2}$  at each of these points (B.J.S. and J.R.S., unpublished observations), which suggests that the increase in  $\dot{V}_{\text{CO}_2}$  reflects an increase in metabolism, rather than a change in the  $\text{CO}_2$ -buffering properties of the blood. Note that an increase in  $\text{CO}_2$  production is in the wrong direction to simply reflect a shift in fuel use from lipid to carbohydrate during this pre-freeze period (Sinclair et al., 2011). We observed the same qualitative pattern of changes in  $\dot{V}_{\text{CO}_2}$  in frogs that were collected at the beginning and end of the winter, and also in frogs that were frozen a second time ( $N=2$ , data not shown), which suggests that this is a repeatable pattern in this species. It will be valuable to measure metabolism in real time in other species for which freezing is associated with mobilisation of glucose [e.g. lizards and earthworms (Costanzo et al., 1995; Holmstrup et al., 1999)]

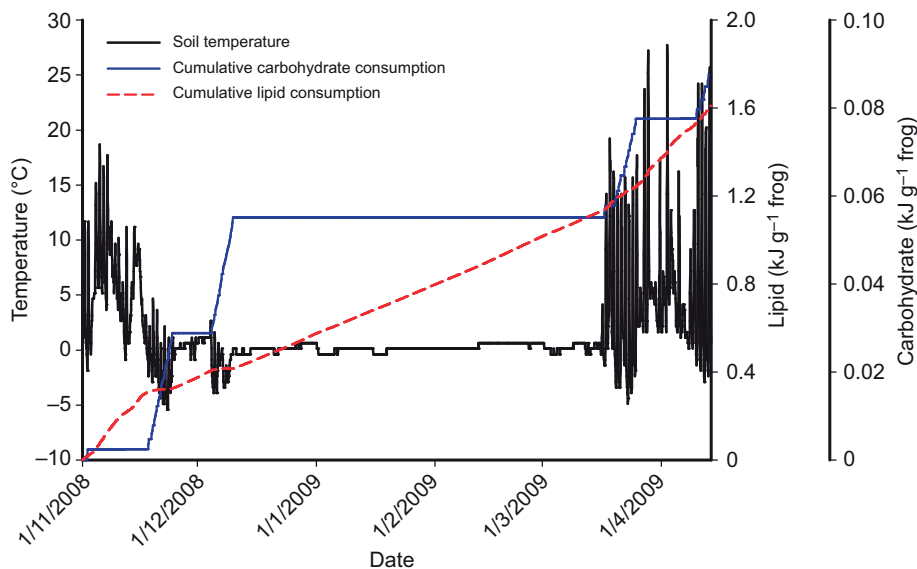


Fig. 5. Example of cumulative overwinter energy use from carbohydrates (grey line) and lipid (dashed line) calculated from microclimate temperatures from Location 2 (black line; see also Tables 2, 3) for a 7.1 g *Rana sylvatica* in the 2008–2009 winter. Note the long period of snow cover, from 10 December to 17 March, when frogs are predicted to have remained unfrozen. Dates are in day/month/year format.

Table 3. Predicted overwinter consumption of energy reserves by a 7.1 g *Rana sylvatica* for two locations in a woodlot near Ottawa in the winter (1 November–16 April) of 2008–2009

	Location 1	Location 2
Number of frog freeze–thaw cycles	16	23
Time spent frozen (h)	270	312
Lipid (base model) (mg triglyceride g <sup>-1</sup> frog)	39.2	42.0
Lipid (mg frog <sup>-1</sup> )	278.2	298.0
CHO (base model) (mg glucose equivalents g <sup>-1</sup> frog)	2.6	3.0
CHO from activity thresholds (mg glucose equivalents g <sup>-1</sup> frog)	0.6	0.7
CHO from freeze–thaw (mg glucose equivalents g <sup>-1</sup> frog)	3.6	5.2
Total CHO (mg glucose equivalents g <sup>-1</sup> frog)	6.9	8.9
Base CHO as a proportion of total CHO	0.39	0.33
Total CHO consumption (mg frog <sup>-1</sup> )	48.7	62.8

See Materials and methods for assumptions of the model. CHO, carbohydrate.

to determine whether the increased CO<sub>2</sub> production with freezing and thawing is a general pattern.

The large increase in  $\dot{V}_{CO_2}$  we observed during cooling (but prior to ice nucleation) was surprising because signal transduction that triggers cryoprotectant synthesis is only initiated by ice nucleation (Storey and Storey, 1985), and *R. sylvatica* held between 0 and –2°C did not mobilise glucose or increase heart rate until ice formation was initiated (Storey and Storey, 1985; Layne et al., 1989). Thus, it does not appear that the observed pre-freeze increase in  $\dot{V}_{CO_2}$  is associated with preparatory physiological processes that are directly linked to known cryoprotection. However, this increase was observed in all frogs, was initiated at above-zero temperatures and was not accompanied by a change in physical activity by the frog. A similar pre-freezing increase in metabolic rate was observed using closed-system respirometry in *Rana arvalis* (Voituron et al., 2009), but these authors also observed an increase in plasma glucose during this time. The increase we observed is unlikely to be due to a threshold response to water loss, as it was dependent on temperature, rather than time in the chamber, and is unlikely to be due to preparatory mobilisation (but not circulation) of glucose from glycogen in the liver, as that process is energetically inexpensive and does not begin until after nucleation. Although we always observed this pre-freeze increase in metabolism at approximately the same temperature in each frog, future real-time measurements of metabolism during cooling from different starting temperatures

will be necessary to determine whether this increase is a response to rapid cooling *per se*, or a response to a specific temperature threshold. In particular, we note that transient receptor potential (TRP) channels are sensitive to changing temperatures, rather than absolute temperature (Voets et al., 2004), so there is a possibility that this mechanism could mediate a metabolic rate to temperature change. Clearly, further work on the dynamic responses of freeze-tolerant vertebrates during the cooling process is necessary to fully understand any preparatory processes.

A release of CO<sub>2</sub> coincident with freezing has been reported in an insect (Sinclair et al., 2004), but in that case the volume of CO<sub>2</sub> released was consistent with expulsion of air from the tracheal system, and in any case, freezing is thought to be largely passive in freeze-tolerant insects (Zachariassen, 1985). In *R. sylvatica*, we detected a sharp increase in  $\dot{V}_{CO_2}$  that was clearly initiated with ice formation, and that persisted beyond the cessation of active ventilation. It is likely that once ventilation ceased, we were observing cutaneous gas exchange, which accounts for a significant proportion of respiration in terrestrial frogs (Feder and Burggren, 1992), and also diffusive exchange with the lungs *via* the nares. We hypothesise that the increase in  $\dot{V}_{CO_2}$  associated with freezing reflects the increased metabolism associated with the production and distribution of cryoprotectants, the costs of the physiological and molecular responses to ice formation, as well as a slight increase in metabolic rate associated

Table 4. Sensitivity of the results of our model of *Rana sylvatica* overwinter energy use to the assumptions made in the model

Adjustment	No. freeze–thaw cycles	Total time spent frozen (h)	Lipid consumption (mg winter <sup>-1</sup> g <sup>-1</sup> frog)	Total CHO consumption (mg glucose equivalents winter <sup>-1</sup> g <sup>-1</sup> frog)
Original model	23	312	41.978	8.85
Pre-freeze increase at 0°C	–	–	–	9.077 (+3%)
$T_c = -0.8$	28 (+22%)	370 (+19%)	41.596 (–1%)	10.725 (+21%)
Melting point = –0.6	–	290 (–7%)	42.134 (+<1%)	8.541 (–3%)
$T_c = -0.8$ , melting point = –0.6	32 (+39%)	336 (+8%)	41.84 (–<1%)	11.148 (+26%)
Threshold for $Q_{10}$ change = 0	–	–	55.326 (+31%)	–
Threshold for $Q_{10}$ change = 8	–	–	43.75 (+4%)	–
$Q_{10} = 2$ throughout	–	–	58.016 (+38%)	12.882 (+46%)
$Q_{10} = 7.97$ throughout	–	–	99.343 (+137%)	–
MR depressed to 50% when frozen	–	–	–	7.374 (–17%)
MR depressed to 5% when frozen	–	–	–	6.047 (–32%)
MR depressed to 0.5% when frozen	–	–	–	5.914 (–33%)

Values in parentheses indicate percent change compared with the original model. All sensitivity analyses were run on microclimate data from Location 2 over the 2008–2009 winter. The original model parameters were: pre-freeze increase = 1.03°C; temperature of crystallisation ( $T_c$ ) = –1.36°C; melting point = –0.16; threshold for  $Q_{10}$  change = +4°C, with  $Q_{10} = 7.97$  below threshold and 2.0 above threshold; no metabolic rate (MR) depression when frozen. ‘–’ indicates no effect of the parameter change.



with the heat released during ice crystallisation (Layne et al., 1989; Storey and Storey, 1996).

The final increase in  $\dot{V}_{CO_2}$  we observed was during thawing, specifically in the period after the temperature of the frog began increasing, indicating that all of the ice had melted. This increase in  $\dot{V}_{CO_2}$  probably reflects the increase in ATP turnover needed to reactivate physiological functions, repair any freeze-induced damage and restore homeostasis (e.g. ion gradients may need to be restored to reactivate nerve transmission and muscle contractility), as well as initiate glucose clearance from the blood and bladder and the resynthesis of liver glycogen (Storey and Storey, 1986; Layne and First, 1991; Costanzo et al., 1997). This period also includes a recovery phase from the hypoxia/anoxia and ischemia stresses experienced by tissues when frozen. Thus, the overshoot will also include the initial costs of processing anaerobic end products (lactate, alanine) that accumulated when frozen, and the costs of macromolecular repair or replacement to redress damage accrued during the freeze–thaw event (Storey and Storey, 1986; Storey and Storey, 1996). It is unlikely that ice formation was maximal after the 12 h cold exposure we applied (Lee et al., 1992), so it may be possible to further investigate the nature of the post-thaw increase in metabolism by manipulating the length of the frozen period, which would allow the cost of thawing to be parsed from the costs incurred while the frog is in the frozen state.

We calculated a  $Q_{10}$  of 7.97 during cooling from +4 to +1°C. This  $Q_{10}$  was consistent with the observed low  $\dot{V}_{CO_2}$  when frozen, and is a little larger than that calculated from measurements presented for *R. arvalis* [~4.5 (Voituron et al., 2009)] but is orders of magnitude lower than the abrupt drops in  $\dot{V}_{CO_2}$  observed to coincide with chill coma onset in insects (Sinclair et al., 2004). The  $Q_{10}$  we measured is nevertheless high – ‘typical’ biological values are 2.0–3.0 (Withers, 1992) – and suggests that the period of cooling below 4°C could include active metabolic suppression, rather than simple Arrhenius effects. Thermal sensitivity of metabolism may change across important biological thresholds (MacMillan et al., 2012), and the  $Q_{10}$  of 7.97 that we measured would be very unusual if it occurred over the whole range of biological temperatures (and, as revealed by our sensitivity analyses, would result in very high energy expenditure at higher temperatures). Therefore, we included a threshold shift in  $Q_{10}$  in our model to reflect the likelihood that thermal sensitivity of metabolism is lower at higher temperatures. We have modelled this shift as a threshold, but it could also be a curvilinear relationship, which would not alter our conclusions. Our assumption of a decrease in the thermal sensitivity of metabolism at higher temperatures was the model parameter with the greatest influence on predicted overwinter lipid use, and therefore on energy use overall. At the extreme, assuming that  $Q_{10}$  is a constant 7.97 more than doubled our estimate of overwinter lipid consumption. Conversely, assuming a much lower thermal sensitivity was predicted to increase both lipid and carbohydrate consumption moderately, the latter because of the lack of metabolic rate suppression at low temperatures.

Our predictions of freezing and thawing in the field suggest that frogs may spend the majority of the winter unfrozen, and that there is inter-location (and undoubtedly inter-annual) variability in the frequency of freeze–thaw events, even within a small geographic area. The number of predicted freeze–thaw cycles is strongly influenced by the temperature of crystallisation used in the model. We used a  $T_c$  of –1.4°C (derived from our empirical measurements); however, frogs in the wild will be in contact with ice in frozen soil or leaf litter, which will nucleate freezing at temperatures up to 1°C warmer (Lee and Costanzo, 1998). A higher  $T_c$  will increase the

number of predicted freeze–thaw cycles, and thus the expected energetic costs associated with freezing, in a linear fashion. However, our sensitivity analyses show that increasing  $T_c$  from –1.4 to –0.8 in our model increased the number of freeze–thaw cycles by only ~20% (Table 4), which is within the range of variability we observed between locations (Table 3). Therefore, a higher  $T_c$  in nature would not substantially alter our conclusions. In addition, we did not place a time constraint on the criteria for identifying a freeze–thaw event. Ice formation and melting take many hours (Fig. 1) (Lee et al., 1992), yet the shortest of the freeze–thaw events we identified was only 1 h. Although it is probable that the costs of freezing and thawing would be lower for shorter events (especially when ice content is low), it is also likely that a certain level of threshold costs exists. Frogs trigger cryoprotectant synthesis within 2 min of nucleation (Storey and Storey, 1985), and the majority of the threshold increase in freezing we observed occurred in the short period after the initiation of ice formation (Fig. 1), suggesting that the initial metabolic costs associated with freezing will have energetic consequences regardless of the amount of ice formed. Manipulating the length of freezing events will allow a finer analysis of the factors that determine variation in the costs of freeze–thaw, and whether short freezing events represent reduced or increased metabolic costs. Variation in intensity of cold exposure has an impact on post-thaw locomotor responses (Layne and Rice, 2003), so it is possible that exposure to higher or lower temperatures would also modify metabolic responses during and after thawing. Future work could also examine the impacts of different minimum temperatures on energetic responses.

In our model, we have assumed that unfrozen overwintering frogs consume mainly lipid, while they consume carbohydrate when frozen and during the freezing process. The latter assumption is supported by the observed accumulation of lactate and alanine as glycolytic end products while frogs are frozen (Storey and Storey, 1986). Although we assume that this transition is a step function, it is quite likely that there will be a period of fuel-use transition from lipid to carbohydrate during the freezing process, and a similar transition during and after thawing. While such transitions could modify the absolute amount of lipid and carbohydrate consumed, they would not substantially alter the conclusion that freeze–thaw thresholds are a primary driver of winter carbohydrate consumption. It is also possible that the pre-freeze increase in  $\dot{V}_{CO_2}$  that we observed could be fuelled by lipid. The pre-freeze increase accounted for a very small amount of overwinter energy consumption in our model (Fig. 4), so we do not expect our conclusions to be altered by metabolic substrate at this threshold. Future fine-scale investigations into the nature of fuel use during these transitions will be necessary to test our assumptions.

Assuming limited protein catabolism and/or gluconeogenesis from protein or other sources over winter, the dichotomy between carbohydrate use when frozen and lipid use when unfrozen means that wood frog energy stores are separated into two pools that respond differently to winter thermal conditions. This is exemplified in our sensitivity analyses, where shifts in some parameters (for example, those that modify the number of freeze–thaw cycles) have a large impact on predicted carbohydrate consumption while leaving predicted lipid consumption largely unchanged. By contrast, thermal sensitivity of metabolism will disproportionately change lipid consumption because it is more important at above-freezing temperatures due to the effects of Jensen’s inequality (Ruel and Ayres, 1999) (Table 4).

It has been predicted that the energy savings of being frozen may be a primary advantage of freeze tolerance (Voituron et al., 2002b).

Comparison of predicted energy use at the two locations is useful because at Location 2 frogs would have experienced more freeze–thaw cycles and spent more time frozen. This difference meant that there was an increase in predicted carbohydrate consumption by frogs at Location 2, but there was no concomitant decrease in predicted lipid consumption. Thus, in this case, the importance of freeze tolerance in energetic savings would depend upon the relative abundance of lipid and carbohydrate reserves, the thermal sensitivity of unfrozen metabolism and the temperatures experienced in the unfrozen state. Metabolic depression when frozen can save significant amounts of energy (Irwin and Lee, 2002; Voituron et al., 2002a; Marshall and Sinclair, 2012) but our sensitivity analyses suggest that metabolic depression when frozen will reduce predicted overwinter carbohydrate consumption by less than one-third, even if metabolism is suppressed by 99.5% (Table 4). This is likely because a significant portion of overwinter carbohydrate consumption occurs as a result of the threshold costs of cooling, freezing and thawing (Fig. 4), which are not modified by metabolic rate depression when frozen. Therefore, if our measurements of metabolic rate during freezing and thawing are representative, it appears that the energetic costs of an active freezing process have the potential to outweigh any energy savings advantages of being frozen, and that freeze tolerance probably evolved as a response to selective pressures other than energy conservation.

*Rana sylvatica* collected from the same location as our individuals have approximately 150 mg carbohydrate g<sup>-1</sup> liver in the autumn and 120 mg carbohydrate g<sup>-1</sup> liver in the spring (Storey and Storey, 1984; Storey and Storey, 1987). Assuming that the liver is 12% of the frog's body mass in autumn and 2.4% in spring (K.B.S., unpublished), and that the liver contains the majority of the frog's store of carbohydrate that fuels overwinter metabolism, we can estimate consumption of 112 mg of carbohydrate in a 7.1 g frog over the course of a winter. Our model predicts overwinter carbohydrate consumption in the region of 44–56% of this value (Table 3), suggesting that our estimates are conservative, but supporting the general form of the model. In particular, this calculation does not account for stores of glycogen in other tissues (Storey and Storey, 1984), which likely fuel a portion of overwinter metabolism as well as metabolic activity in the spring. Lipid stores are variable among individuals (K.B.S., unpublished), but predictions of overwinter lipid consumption could, in future, be tested using non-invasive techniques on individuals, for example with quantitative magnetic resonance spectroscopy (Guglielmo et al., 2011). The stoichiometry underlying our predictions of carbohydrate consumption assumes aerobic respiration throughout the winter. Although gas exchange is possible across the skin and mucosal membranes, frozen frogs accumulate anaerobic end products, which will be fuelled by carbohydrate metabolism, but not reflected immediately in CO<sub>2</sub> release (Storey and Storey, 1984; Storey and Storey, 1986). This anaerobic metabolism will not be included in energy consumption in our model, unless the increase in metabolism during and after thawing is reflective of the catabolism of anaerobic end products accumulated when the frog was frozen. Thus, our assumptions may lead to an underestimate of carbohydrate consumption.

Finally, we point out that our microclimate recordings are only an approximation of frog temperature. Our woodlot location is slightly warmer than the temperatures recorded at a nearby meteorological station, while snow cover and the effect of insolation on leaf litter temperature suggest that meteorological measurements would be a poor approximation of frog temperature (supplementary

material Fig. S1). Also, our measurements assume that there is no thermal inertia in the frogs' cooling and warming rates. This thermal inertia may be particularly important during the endothermic stage of ice melting, which means that any brief periods of thawing may not lead to the frog thawing completely. We did not explore whether there are additional energetic consequences for partial thawing or re-freezing. We suggest that future studies may aim to obtain a better estimate of the temperatures actually experienced by the frogs, either by housing data loggers in models matched for the thermal mass of the animal (e.g. Hertz, 1992), or by implanting data loggers in overwintering frogs (e.g. Rausch et al., 2008).

### Energy use in a changing climate

The present results and predictions from our model can be used to illustrate some important potential impacts of winter climate change on *R. sylvatica*, and other species that have an active response to internal ice formation. Either higher temperatures or a reduction in precipitation could decrease the depth or persistence of the snow pack and lead to an increase in freeze–thaw cycles (Henry, 2008), resulting in increased carbohydrate consumption over the winter. The timing of these freeze–thaw cycles is important: if there are more freeze–thaw cycles during the early winter, due to a delay in the accumulation of snow cover, this could lead to a premature reduction in carbohydrate reserves, and thus depletion of both metabolic fuel and cryoprotectants. Such a scenario would be accompanied by greater exposure to high temperatures, leading also to higher lipid consumption. Changing autumn conditions (for example, a shift in the timing of winter snow accumulation in the Ottawa location where our frogs were collected) could lead to increased carbohydrate consumption due to an increase in the number and frequency of freeze–thaw cycles, as well as an increase in lipid consumption because of warmer non-freezing conditions. Similar implications of climate change for overwinter energy use are also predicted to have an impact on some capital-breeding insects, such as overwintering butterflies (Williams et al., 2012a; Williams et al., 2012b) and hibernating mammals (Humphries et al., 2002). In the case of *R. sylvatica*, many of our predictions may be tested through field studies across a wider geographical area representing a range of winter temperatures and snow cover situations.

### LIST OF SYMBOLS AND ABBREVIATIONS

$Q_{10}$	thermal sensitivity coefficient
RQ	respiratory quotient
$T$	temperature
$T_c$	temperature of crystallisation
$\dot{V}_{CO_2}$	rate of carbon dioxide production

### ACKNOWLEDGEMENTS

Thanks to Jan Storey and two anonymous referees for insightful comments on an earlier version of the manuscript.

### FUNDING

This research was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Undergraduate Research Award to J.R.S.; an NSERC Discovery Grant, the Canadian Foundation for Innovation and an Ontario Early Researcher Award to B.J.S.; an Ontario Graduate Scholarship to C.M.W.; NSERC doctoral scholarships to K.E.M. and H.A.M.; and an NSERC Discovery grant and Canada Research Chair to K.B.S.

### REFERENCES

- Baldwin, R. F., Calhoun, A. J. K. and deMaynadier, P. G. (2006). Conservation planning for amphibian species with complex habitat requirements: a case study using movements and habitat selection of the wood frog *Rana sylvatica*. *J. Herpetol.* **40**, 442–453.

- Costanzo, J. P., Lee, R. E., Jr and Lortz, P. H. (1993). Physiological responses of freeze-tolerant and -intolerant frogs: clues to evolution of anuran freeze tolerance. *Am. J. Physiol.* **265**, R721-R725.
- Costanzo, J. P., Grenot, C. and Lee, R. E., Jr (1995). Supercooling, ice inoculation and freeze tolerance in the European common lizard, *Lacerta vivipara*. *J. Comp. Physiol. B* **165**, 238-244.
- Costanzo, J. P., Callahan, P. A., Lee, R. E., Jr and Wright, M. F. (1997). Frogs reabsorb glucose from urinary bladder. *Nature* **389**, 343-344.
- Feder, M. E. and Burggren, W. W. (1992). *Environmental Physiology of the Amphibians*. Chicago, IL: University of Chicago Press.
- Fitzpatrick, L. C. (1976). Life-history patterns of storage and utilization of lipids for energy in amphibians. *Am. Zool.* **16**, 725-732.
- Guglielmo, C. G., McGuire, L. P., Gerson, A. R. and Seewagen, C. L. (2011). Simple, rapid, and non-invasive measurement of fat, lean, and total water masses of live birds using quantitative magnetic resonance. *J. Ornithol.* **152**, 75-85.
- Hansen, L. L., Westh, P., Wright, J. C. and Ramløv, H. (2006). Metabolic changes associated with active water vapour absorption in the mealworm *Tenebrio molitor* L. (Coleoptera, Tenebrionidae): a microcalorimetric study. *J. Insect Physiol.* **52**, 291-299.
- Henry, H. A. L. (2008). Climate change and soil freezing dynamics: historical trends and projected changes. *Clim. Change* **87**, 421-434.
- Hertz, P. E. (1992). Temperature regulation in Puerto Rican *Anolis* lizards – a field test using null hypotheses. *Ecology* **73**, 1405-1417.
- Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptation*. New York, NY: Oxford University Press.
- Holmstrup, M., Costanzo, J. P. and Lee, R. E., Jr (1999). Cryoprotective and osmotic responses to cold acclimation and freezing in freeze-tolerant and freeze-intolerant earthworms. *J. Comp. Physiol. B* **169**, 207-214.
- Hui, W., Gel, Y. R. and Gastwirth, J. L. (2008). lawstat: an R package for law, public policy and biostatistics. *J. Stat. Softw.* **28**, 1-26.
- Humphries, M. M., Thomas, D. W. and Speakman, J. R. (2002). Climate-mediated energetic constraints on the distribution of hibernating mammals. *Nature* **418**, 313-316.
- Irwin, J. T. and Lee, R. E., Jr (2000). Mild winter temperatures reduce survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae). *J. Insect Physiol.* **46**, 655-661.
- Irwin, J. T. and Lee, R. E., Jr (2002). Energy and water conservation in frozen vs. supercooled larvae of the goldenrod gall fly, *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae). *J. Exp. Zool.* **292**, 345-350.
- Irwin, J. T., Costanzo, J. P. and Lee, R. E., Jr (2003). Postfreeze reduction of locomotor endurance in the freeze-tolerant wood frog, *Rana sylvatica*. *Physiol. Biochem. Zool.* **76**, 331-338.
- Layne, J. R., Jr (2000). Postfreeze O<sub>2</sub> consumption in the wood frog (*Rana sylvatica*). *Copeia* **2000**, 879-882.
- Layne, J. R., Jr and First, M. C. (1991). Resumption of physiological functions in the wood frog (*Rana sylvatica*) after freezing. *Am. J. Physiol.* **261**, R134-R137.
- Layne, J. R., Jr and Kefauver, J. (1997). Freeze tolerance and postfreeze recovery in the frog *Pseudacris crucifer*. *Copeia* **1997**, 260-264.
- Layne, J. R., Jr and Lee, R. E., Jr (1995). Adaptations of frogs to survive freezing. *Clim. Res.* **5**, 53-59.
- Layne, J. R., Jr and Rice, M. E. (2003). Postfreeze locomotion performance in wood frogs (*Rana sylvatica*) and spring peepers (*Pseudacris crucifer*). *Can. J. Zool.* **81**, 2061-2065.
- Layne, J. R., Jr, Lee, R. E., Jr and Heil, T. L. (1989). Freezing-induced changes in the heart rate of wood frogs (*Rana sylvatica*). *Am. J. Physiol.* **257**, R1046-R1049.
- Lee, R. E., Jr and Costanzo, J. P. (1998). Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *Annu. Rev. Physiol.* **60**, 55-72.
- Lee, R. E., Jr, Costanzo, J. P., Davidson, E. C. and Layne, J. R., Jr (1992). Dynamics of body water during freezing and thawing in a freeze-tolerant frog (*Rana sylvatica*). *J. Therm. Biol.* **17**, 263-266.
- Lighton, J. R. B. and Turner, R. J. (2004). Thermolimit respirometry: an objective assessment of critical thermal maxima in two sympatric desert harvester ants, *Pogonomyrmex rugosus* and *P. californicus*. *J. Exp. Biol.* **207**, 1903-1913.
- Long, D. R. (1987). A comparison of energy substrates and reproductive patterns of two anurans. *Acris crepitans* and *Bufo woodhousei*. *Comp. Biochem. Physiol.* **87A**, 81-91.
- MacMillan, H. A., Williams, C. M., Staples, J. F. and Sinclair, B. J. (2012). Metabolism and energy supply below the critical thermal minimum of a chill-susceptible insect. *J. Exp. Biol.* **215**, 1366-1372.
- Marshall, K. E. and Sinclair, B. J. (2012). Threshold temperatures mediate the impact of reduced snow cover on overwintering freeze-tolerant caterpillars. *Naturwissenschaften* **99**, 33-41.
- McLister, J. D. (2003). The metabolic cost of amplexus in the grey tree frog (*Hyla versicolor*): assessing the energetics of male mating success. *Can. J. Zool.* **81**, 388-394.
- Muir, T. J., Costanzo, J. P. and Lee, R. E., Jr (2008). Metabolic depression induced by urea in organs of the wood frog, *Rana sylvatica*: effects of season and temperature. *J. Exp. Zool.* **A 309**, 111-116.
- R Development Core Team (2010). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Rausch, C. M., Starkweather, P. L. and van Breukelen, F. (2008). One year in the life of *Bufo punctatus*: annual patterns of body temperature in a free-ranging desert anuran. *Naturwissenschaften* **95**, 531-535.
- Regosin, J. V., Windmiller, B. S. and Reed, J. M. (2003). Terrestrial habitat use and winter densities of the wood frog (*Rana sylvatica*). *J. Herpetol.* **37**, 390-394.
- Rexer-Huber, K. M. J., Bishop, P. J. and Wharton, D. A. (2011). Skin ice nucleators and glycerol in the freezing-tolerant frog *Litoria ewingii*. *J. Comp. Physiol. B* **181**, 781-792.
- Ruel, J. J. and Ayres, M. P. (1999). Jensen's inequality predicts effects of environmental variation. *Trends Ecol. Evol.* **14**, 361-366.
- Sinclair, B. J. (2001). Biologically relevant environmental data: macros to make the most of microclimate recordings. *Cryo Letters* **22**, 125-134.
- Sinclair, B. J., Klok, C. J. and Chown, S. L. (2004). Metabolism of the sub-Antarctic caterpillar *Pringleophaga marioni* during cooling, freezing and thawing. *J. Exp. Biol.* **207**, 1287-1294.
- Sinclair, B. J., Bretman, A., Tregenza, T., Tomkins, J. L. and Hosken, D. J. (2011). Metabolic rate does not decrease with starvation in *Gryllus bimaculatus* when changing fuel use is taken into account. *Physiol. Entomol.* **36**, 84-89.
- Storey, J. M. and Storey, K. B. (1985). Triggering of cryoprotectant synthesis by the initiation of ice nucleation in the freeze tolerant frog, *Rana sylvatica*. *J. Comp. Physiol. B* **156**, 191-195.
- Storey, K. B. (2004). Strategies for exploration of freeze responsive gene expression: advances in vertebrate freeze tolerance. *Cryobiology* **48**, 134-145.
- Storey, K. B. (2008). Beyond gene chips: transcription factor profiling in freeze tolerance. In *Hypometabolism in Animals: Hibernation, Torpor and Cryobiology* (ed. B. G. Lovegrove and A. E. McKechnie), pp. 101-108. Pietermaritzburg, South Africa: Interpak Books.
- Storey, K. B. and Storey, J. M. (1984). Biochemical adaptation for freezing tolerance in the wood frog, *Rana sylvatica*. *J. Comp. Physiol. B* **155**, 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**, 720-726.
- Storey, K. B. and Storey, J. M. (1992). Natural freeze tolerance in ectothermic vertebrates. *Annu. Rev. Physiol.* **54**, 619-637.
- Storey, K. B. and Storey, J. M. (1996). Natural freezing survival in animals. *Annu. Rev. Ecol. Syst.* **27**, 365-386.
- Tattersall, G. J. and Ultsch, G. R. (2008). Physiological ecology of aquatic overwintering in ranid frogs. *Biol. Rev. Camb. Philos. Soc.* **83**, 119-140.
- Voets, T., Droogmans, G., Wissenbach, U., Janssens, A., Flockerzi, V. and Nilius, B. (2004). The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature* **430**, 748-754.
- Voituron, Y., Verdier, B. and Grenot, C. (2002a). The respiratory metabolism of a lizard (*Lacerta vivipara*) in supercooled and frozen states. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **283**, R181-R186.
- Voituron, Y., Mouquet, N., de Mazancourt, C. and Clobert, J. (2002b). To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms. *Am. Nat.* **160**, 255-270.
- Voituron, Y., Paaschburg, L., Holmstrup, M., Barré, H. and Ramløv, H. (2009). Survival and metabolism of *Rana arvalis* during freezing. *J. Comp. Physiol. B* **179**, 223-230.
- Williams, C. M., Marshall, K. E., MacMillan, H. A., Dzurisin, J. D. K., Hellmann, J. J. and Sinclair, B. J. (2012a). Thermal variability increases the impact of autumnal warming and drives metabolic depression in an overwintering butterfly. *PLoS ONE* **7**, e34470.
- Williams, C. M., Hellmann, J. J. and Sinclair, B. J. (2012b). Lepidopteran species differ in susceptibility to winter warming. *Clim. Res.* **53**, 119-130.
- Withers, P. C. (1992). *Comparative Animal Physiology*. Fort Worth, TX: Saunders College Publishing.
- Wolanczyk, J. P., Storey, K. B. and Baust, J. G. (1990). Ice nucleating activity in the blood of the freeze-tolerant frog, *Rana sylvatica*. *Cryobiology* **27**, 328-335.
- Zachariassen, K. E. (1985). Physiology of cold tolerance in insects. *Physiol. Rev.* **65**, 799-832.
- Zhang, J. and Storey, K. B. (2012). Cell cycle regulation in the freeze tolerant wood frog, *Rana sylvatica*. *Cell Cycle* **11**, 1727-1742.