RESEARCH ARTICLE

Glycogen, not dehydration or lipids, limits winter survival of side-blotched lizards (*Uta stansburiana*)

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SUMMARY

Climate change is causing winters to become milder (less cold and shorter). Recent studies of overwintering ectotherms have suggested that warmer winters increase metabolism and decrease winter survival and subsequent fecundity. Energetic constraints (insufficient energy stores) have been hypothesized as the cause of winter mortality but have not been tested explicitly. Thus, alternative sources of mortality, such as winter dehydration, cannot be ruled out. By employing an experimental design that compared the energetics and water content of lizards that died naturally during laboratory winter with those that survived up to the same point but were then sacrificed, we attempt to distinguish among multiple possible causes of mortality. We test the hypothesis that mortality is caused by insufficient energy stores in the liver, abdominal fat bodies, tail or carcass or through excessive water loss. We found that lizards that died naturally had marginally greater mass loss, lower water content, and less liver glycogen remaining than living animals sampled at the same time. Periodically moistening air during winter reduced water loss, but this did not affect survival, calling into question dehydration as a cause of death. Rather, our results implicate energy limitations in the form of liver glycogen, but not lipids, as the primary cause of mortality in overwintering lizards. When viewed through a lens of changing climates, our results suggest that if milder winters increase the metabolic rate of overwintering ectotherms, individuals may experience greater energetic demands. Increased energy use during winter may subsequently limit individual survival and possibly even impact population persistence.

Key words: climate change, survival, overwintering, energetics, triacylglycerides, water loss.

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INTRODUCTION

Biologically relevant alterations to the environment linked to climate change include longer growing seasons (e.g. Schwartz and Reiter, 2000; Bradshaw and Holzapfel, 2001; Beaumont et al., 2006; Schwartz et al., 2006), higher daily and annual maximum and minimum temperatures (e.g. DeGaetano, 1996; Easterling et al., 1997; DeGaetano and Allen, 2002), redistribution of moisture patterns (e.g. Groisman et al., 1999) or a combination of these depending on regional or local effects (Easterling et al., 2000; Meehl et al., 2000). Previous studies have indicated that for many regions of the Earth, summers are not necessarily hotter but winters are becoming milder (DeGaetano, 1996; Easterling et al., 1997; Easterling et al., 2000). Such environmental alterations have the potential to affect seasonal ectotherms, which typically have a metabolic rate dependent upon temperature. For example, studies of ectotherms, such as insects and lizards, have suggested that colder winters increase energy conservation (Pullin and Bale, 1989b) and winter survival (Pullin and Bale, 1989a; Irwin and Lee, 2000; Zani, 2008) as well as subsequent reproduction (Irwin and Lee, 2000). Thus, colder winter environments may actually increase survival in nature, as has been reported for at least one lizard species; populations of side-blotched lizards (*Uta stansburiana*) from higher latitudes have lower overwinter mortality

than populations from lower latitudes (Wilson and Cooke, 2004). In the absence of any behavioral shift to cooler microenvironments during the winter, warmer winters due to climate change may cause elevated metabolic rates (forcing animals to rely more heavily on stored energy) and reduce both survivorship (Wilson and Cooke, 2004; Zani, 2008) and subsequent fecundity (Irwin and Lee, 2003; Williams et al., 2003).

A recent study reported that winter survival of lizards in the lab was related both to body size (bigger lizards survived longer) and temperature (those in colder conditions survived longer) (Zani, 2008). Consistent with previous research, these results were interpreted as due to energetic constraints; larger lizards are able to store more energy, and colder environments cause lizards to metabolize stored energy at a slower rate, both of which lead to increased survival. However, previous research has not attempted to determine if the hypothesized constraints were due to energetic limitations, such as the amount of stored carbohydrate (i.e. glycogen) or lipid (i.e. triacylglyceride). Considering that lizards store energy in several locations in the body (glycogen and triacylglyceride in liver and muscle, triacylglyceride in distinct abdominal fat bodies), it is possible that any one of these tissues represents the critical limit for survival. Alternatively, the survival constraint may be

caused by some other factor, such as hydration (i.e. water content). The vital limits for water loss have been documented for many reptiles (e.g. see Hall, 1922; Heatwole and Veron, 1977; Munsey, 1972), but the role of hydration for winter survival appears to be less well studied and has focused more on the relationship of hydration to thermal tolerances (Costanzo et al., 2001; Baker et al., 2003; Dinkelacker et al., 2004).

In the present study, we attempt to address the question of whether winter survival in ectotherms relates to energetics or some other cause, such as hydration. However, because constraints on survival could be caused by limitations in any one tissue/organ or the whole organism, or by any one of several vital stores (e.g. glycogen, triacylglyceride or water), we sought to test among the multiple alternative hypotheses by exposing animals to a simulated winter environment. We hypothesize that lizards experiencing winter environments are able to survive for as long as some limiting resource (energy, moisture) is available, that the amount of resource stores is positively correlated with body size, and that the rate of resource use is positively related to temperature. Thus, as winter progresses, animals slowly utilize their vital stores in a temperature-dependent manner. As such, we predicted that the critical limiting resource would slowly decrease in quantity over the course of the winter as it was consumed and/or lost (i.e. a negative relationship with time). This drawdown of resources continues throughout winter to the point where animals reach their vital (i.e. survival) limits, at which point they die (Fig. 1). Yet animals still alive at this same point in time should have significantly greater amounts of the vital store, which is why, of course, they have not yet died. Specifically, we test the possibility that the limiting factor for survival is organ energy content (measured as glycogen or triacylglyceride in the liver, fat bodies or tail where appropriate), carcass lipid content (measured as total lipids in the remaining carcass) or hydration (measured as carcass water content).

MATERIALS AND METHODS
Study organism and study site
Common side-blotched lizards, *Uta stansburiana* (Baird and Girard), are small [~40–60 mm adult snout–vent length (SVL)], diurnal phrynosomatid lizards common in western North America. They range from the southern tip of Baja California, Mexico, north to low-elevation areas of the Columbia River Basin in Washington State, USA. Lizards for this study were collected at Wrights Point, 20 km south of the high-desert town of Burns, in eastern Oregon, USA (43.437°N, 118.928°W, 1318 m elevation), which is at the northern edge of the Great Basin Desert. Wrights Point is a large lava flow (~15 km long, ~0.25 km wide, ~75 m high) that runs east–west in the Malheur Basin with a series of slowly eroding south-facing cliffs that have created boulder-strewn slopes. This population is among the highest in elevation in the Pacific Northwest (Nussbaum et al., 1983) (P.A.Z., personal observation) and likely persists because of the quality of the hibernacula at this site as well as the surrounding Malheur wetlands. In the fall, lizards abandon their home ranges and congregate around hibernacula on the vertical cliff faces. Hibernacula sites all appear to share certain characteristics (P.A.Z., personal observation) [see p. 243 in Nussbaum et al. (Nussbaum et al., 1983)]: large thermal mass (i.e. small rock outcrops never appear to be hibernacula), access to crevices >1 m deep (in some instances, this is by multiple cracks while in others there appears to be one small entrance) and potential for winter sun to penetrate those crevices to some degree (cracks shaded by other rocks or facing generally north do not appear to be used). While *U. stansburiana* can be active year-round at low elevations in the south (Cowles, 1941), in the north their activity is restricted to a relatively
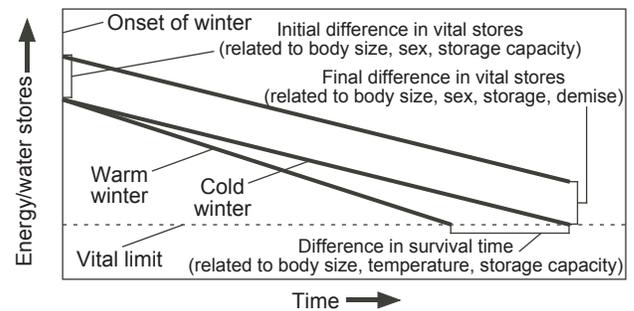

Fig. 1. Model showing experimental design and predicted effects of overwintering on lizard vital stores (energy or hydration). We hypothesize that at the onset of winter larger animals have greater stores of energy and/or water than smaller animals, that these vital stores are utilized (i.e. decrease) as winter progresses, that the rate of utilization is temperature dependent, and that at death an individual will have utilized its store of that vital resource but that animals still alive after the same length of winter will have significantly greater amounts of that vital store. Thus, the predicted effects are related to body size, sex, initial vital storage capacity, winter temperature and whether the individual survives or has reached its vital limit.

short favorable growing season from late March or early April through October (~200 days) (Nussbaum et al., 1983). Thus, lizards must survive an unfavorable season of ≥150 days by retreating ≥1 m into deep cracks and crevices in the cliffs on which they live.

Laboratory acclimation and winter survival
Previous field observations (by P.A.Z.) indicate that lizards remain active in the fall during sunny weather until daily maximum temperature drops below ~8°C, which typically occurs in late October or early November. To study the effects of winter temperatures on survivorship, we collected a sample of 100 lizards (both adults and juveniles) from Wrights Point from 6–12 October 2007. Lizards were shipped overnight to Lafayette College in Pennsylvania, USA. The use of vertebrate animals was approved by the Lafayette College Animal Care and Use Committee. Animals were maintained in common-garden short-day conditions (10 h:14 h L:D) in 1-m-diameter cages constructed of 35-cm-high aluminum flashing walls with 10 cm of sand as substrate and cinder-block pieces as retreats. Each cage was heated at one end by a 120-W incandescent bulb suspended 50 cm above the sand and lit by fluorescent lights suspended 1 m above the sand. The room warmed each day to a high of ~25°C and cooled to 8–10°C at night. Lizards were fed daily with crickets and vestigial-wing fruit flies and watered *ad libitum*. In this way, lizards were maintained for approximately 2 months to acclimate to laboratory conditions. To simulate the onset of the unfavorable season, the photo- and thermo-period were reduced by 15 min per week. In addition, the daily high temperature was reduced by ~2°C per week. For one week prior to the start of the experiment, lizards were watered, but not fed, to allow for purging of gut contents.

On the first day of the experiment (25 December 2007), lizards (*N*=88) were paired with a same-sex animal that most closely matched its mass. These pairs were then randomly assigned (using Microsoft Excel) to a warm (8°C) or cold (2°C) treatment group with the caveat of equal distribution of males and females in each treatment. Within each treatment, lizards were randomly assigned to one of three replicate 7-liter plastic boxes filled with 2.5 liters of sand. Thus, each box contained 15 lizards. Boxes were then stacked inside a constant-temperature incubator set at either 2°C or 8°C and rotated within each incubator every 12 h to minimize temperature differences between replicates. These treatment temperatures were

chosen because they represent conditions at which lizards enter torpor and remain inactive (see also Zani, 2008).

Throughout the experiment, lizards were checked for mortality every 12 h. Survival was assayed by prodding each lizard with a finger or blunt probe; lizards that moved in response were deemed alive. As a means of determining rate of mass loss, lizards were reweighed every two weeks. When a lizard died, it was weighed on an Acculab PP2060D electronic balance (Edgewood, NY, USA) to 0.01 g. This mass was subtracted from the initial mass to calculate mass lost. We then dissected out the entire liver and both abdominal fat bodies as well as the anterior-most 10 mm of the tail (beginning just posterior to testes in males). These organs were weighed on a Sartorius balance (Brinkmann Instruments, Westbury, NY, USA) to 0.001 g and frozen individually at –80°C for subsequent analysis. In addition, the other lizard of the pair (same sex, similar mass) was immediately weighed and killed by decapitation. Because lizards that died naturally had potentially desiccated in their cages for up to 12 h, we randomly assigned each lizard that we sacrificed a wait time (1–12 h) after it was decapitated. Thus, each lizard was placed back in the appropriate cold incubator for that time period before it was dissected. Lizard carcasses (minus the liver, fat bodies and tail base) were weighed and dried in an oven at 60°C until they reached constant mass to determine water content. Dried carcasses were subsequently used for gravimetric lipid extraction (see below). For logistical reasons, the experiment was terminated after 149 days of 'winter' (21 May 2008). Surviving animals were then mailed overnight to Burns, OR, USA and released into a 100 m^2 semi-natural enclosure where they were allowed to live out the remainder of their lives.

HPTLC analysis

Tissue samples of <100 mg in blotted wet mass were homogenized in 2 ml of chloroform/methanol (2:1 by volume) in a 7 ml capacity Wheaton (Millville, NJ, USA) glass homogenizer. This solvent/sample ratio assured complete extraction of the lipids (Folch et al., 1957). The Folch wash (0.88% KCl, w/v, in deionized water) was used in a ratio of 4 parts to 1 part salt solution to remove non-lipophilic material. Samples were vortex-mixed for 30 s, and the top (aqueous) layer removed and discarded. The samples were dried in a warm water bath (40–60°C) using a stream of nitrogen gas and stored at –20°C until use. To obtain appropriate densitometry scan areas within the calibration curves, samples were reconstituted prior to high-performance thin-layer chromatography (HPTLC) tissue analysis in 1.5–10 ml of chloroform/methanol (2:1), as necessary.

The neutral lipid standard, non-polar lipid mixture B (Matreya, Inc., Pleasant Gap, PA, USA), was dissolved in chloroform/methanol (2:1). The standard contained 20.0% each of cholesterol, oleic acid, triolein, methyl oleate and cholesteryl oleate and a total lipid concentration of 25.0 mg ml^{-1}. This standard was used to represent the sample neutral classes of free sterols, free fatty acids, triacylglycerols (hereafter, TG), methyl esters and steryl esters, respectively, and was prepared at a concentration of 0.200 μg μl^{-1}.

HPTLC analysis was performed on 10×20 cm HPTLC-HLF silica gel plates (Analtech, Inc., Newark, DE, USA), which contained 19 scored lanes and a concentration zone spotting area. Before use, plates were prewashed by development to the top with dichloromethane/methanol (1:1) and dried with a stream of air.

Standard and reconstituted sample solutions (2, 4, 8 and 16 μl) were applied to the concentration zone of separate lanes on the HPTLC plates using a 10-μl (Drummond, Broomall, PA, USA) digital microdispenser. We developed plates to a distance of 8 cm beyond the concentration-zone silica gel interface with 25 ml of petroleum ether / diethyl ether / glacial acetic acid (80:20:1) mobile

phase (Mangold, 1969). This development was carried out in a twin trough thin layer chromatography (TLC) chamber (Camag, Wilmington, NC, USA) containing a saturation pad (Analtech, Inc.). Prior to development, the chamber was equilibrated with the mobile phase for 20 min. Development, which required approximately 8–9 min, was carried out at 21°C and a relative humidity of ~25%.

After development, plates were dried with a stream of cool air from a hair dryer, sprayed with 5% ethanolic phosphomolybdic acid solution and heated on a Camag plate heater at 115°C for 10 min to detect neutral lipids as blue zones on a yellow background. For a single liver sample, a plate was developed in hexane / petroleum ether / diethyl ether / glacial acetic acid (50:20:5:1) mobile phase (Smith et al., 1995) to confirm or reject the presence of the fast-moving methyl ester and steryl ester zones. This solvent system is excellent for resolving neutral lipids that migrate at or near the mobile-phase front in the Mangold solvent system.

Quantitative densitometric analysis was carried out using a TLC Scanner II (Camag) with the tungsten light source set at a wavelength of 610 nm, slit width 4, slit length 4, and scanning rate of 4 mm s^{-1}. The CATS-3 software automatically generated polynomial calibration curves (standard zone masses *versus* peak areas) and interpolated sample masses based on their peak areas. We calculated the percentage by mass of lipid in each tissue sample using the equation:

$$\% \text{ Neutral lipid} = (w \times R \times \text{dilution factor} \times 100) / \text{initial tissue sample mass} , \qquad (1)$$

where w is lipid mass (μg) of sample interpolated from the calibration curve, R is reconstituted volume (μl)/spotted volume (μl), and initial sample mass is measured in μg. For samples that were diluted or concentrated to obtain bracketed scan areas within the calibration curve, an appropriate dilution factor was included in the calculation of percent neutral lipid.

Liver glycogen

Liver glycogen content was measured in ~25–75 mg subsamples of liver. The tissue samples were homogenized in 800 μl ice-cold 0.6 mol l^{-1} perchloric acid using a Tissue Tearor (Biospec Products Inc., Bartlesville, OK, USA). Most of the homogenate (700 μl) was centrifuged and the supernatant neutralized with a half-volume of 1 mol l^{-1} sodium bicarbonate. After another centrifugation, the supernatant was stored at –80°C until used for assays of free glucose using the glucose oxidase procedure (GAGO20; Sigma-Aldrich, St Louis, MO, USA). A 100 μl subsample of the original homogenate was neutralized with 50 μl of 1 mol l^{-1} sodium bicarbonate, then exposed to amyloglucosidase (A1602; Sigma-Aldrich) in acetate buffer (119 μmol l^{-1} sodium acetate, 77 μmol l^{-1} acetic acid, pH 4.8) and incubated at 40°C for 2 h to allow the conversion of glycogen into glucose. Enzyme activity was stopped by the addition of 250 μl of 0.6 mol l^{-1} perchloric acid, neutralized with 375 μl of 1 mol l^{-1} sodium bicarbonate, and centrifuged. We used a glucose assay kit (GAGO20; Sigma-Aldrich) to assay the supernatant for the total glucose in the sample, which includes both the free glucose in the tissue plus the glucose liberated from the stored glycogen by amyloglucosidase. Glycogen concentration was calculated as the total glucose in the digested sample minus the free glucose in the original homogenate. Total glycogen content of the liver was calculated as total liver mass multiplied by glycogen concentration. Glycogen concentration is given in glucose units relative to liver size.

Carcass lipid extraction

Dried lizard carcasses were ground into a fine powder to extract the remaining lipids by chloroform/methanol extraction (Folch et

al., 1957). To accomplish this, carcasses were homogenized using a coffee grinder, divided into three roughly equal parts (subsamples) and reweighed. Each part was then separately ground by hand in a glass grinder with 5–10 ml of chloroform/methanol (2:1), which extracts both polar and neutral lipids. This mixture was passed through a glass pipet filled with glass wool and rinsed with an additional 10–15 ml of chloroform/methanol into a pre-weighed glass vial to separate the soluble lipids from the solid material. The samples were dried in a hood in a warm water bath (40–60°C) for 18–24 h until all chloroform/methanol was evaporated and the sample achieved a constant mass. The vial was reweighed to determine the total mass of lipids and compared to the initial sample mass to determine proportion of lipids. The amount of lipid in the three subsamples was averaged for each individual for analyses and scaled up to determine whole-carcass lipid content.

Total animal energy content

Total energy content of each animal was calculated by first determining the energy content of the glycogen and triacylglyceride (by far the major storage products) in each organ (in kcal) using values from extractions. We also used the total of lipids from gravimetric extractions of the carcass. We summed the triacylglyceride contents from the liver, fat bodies, tail and carcass and converted this total to energy units by assuming an energetic content of $9.4\,\text{kcal}\,\text{g}^{-1}$ (Schmidt-Nielsen, 1997). We added to this the energetic content contributed by liver glycogen, which was converted using an estimate of $4.2\,\text{kcal}\,\text{g}^{-1}$ (Schmidt-Nielsen, 1997).

Winter hydration experiment

We performed a second experiment to determine the importance of hydration for lizard overwinter survival and mass retention. For this experiment, eggs from gravid females from Wrights Point were collected during the breeding season of 2008, incubated and hatched in the laboratory [see Zani (Zani, 2008) for detailed methods]. Hatchlings were maintained in the lab (as above) on a long-day photoperiod (18 h:6 h light:dark) and 'summer' temperature range (daily high of 35°C, low of 20°C). Lizards were maintained at these conditions (fed and watered *ad libitum*) until early September, when photoperiod was reduced by 15 min per week and daily temperatures were reduced by 2°C per week. Starting on 2 October, lizards were watered, but not fed, for two weeks to allow them to clear their guts before being exposed to 'winter'. On 16 October, lizards (N=85) were weighed using a Denver Instruments (Bohemia, NY, USA) MXXX-123 electronic balance to 0.001 g, randomly assigned to one of two groups (hydration, no hydration), added to six replicate 6.7 liter plastic boxes filled with 2.5 liters of sand (14 per box) and placed in two constant-temperature incubators set for 2°C. Every 72 h, the lids of the plastic boxes in the hydration treatment were misted with water; lizards were not directly misted to avoid saturating the sand substrate. While we did not measure relative humidity, beads of water on the lid of the cage throughout the experiment indicated it was near saturation. Once every 24 h, we checked lizards for mortality using the same probing technique as above and rotated the plastic boxes both within and between incubators to minimize position effects. Every two weeks we reweighed all animals to determine rate of mass loss. This experiment was terminated after 78 days (2 January 2009).

Statistical analyses

All statistical analyses were conducted using JMP v.7.0.1 (SAS Institute Inc., Pacific Grove, CA, USA) for Macintosh computer. For organ size (e.g. liver mass, fat-body mass) and energy-content traits (e.g. liver glycogen content, total energy content), we were not interested in comparing actual organ sizes or energy contents but in mass-specific values (i.e. values relative to body size). Thus, we first divided organ size or energy content by body mass at death or organ size, respectively. Next, to achieve normality, we $\log_{10}$-transformed length of survival, all body-mass and organ-mass traits (e.g. initial mass, liver mass) and all energy-content traits (e.g. liver glycogen content, carcass lipid content, total energy content) but not percent mass loss or water content. However, we report only non-log-transformed data in tables for ease of interpretation. All data are reported as means ± 1 s.e.m.

First, we conducted analyses to determine if survival was related to initial body size and temperature treatment. For these analyses, we excluded animals that were sacrificed as part of this experiment. Thus, only animals that died naturally or survived the length of the experiment (149 days) were included in these analyses. We began with a logistic regression on survival (yes/no) in which initial mass, treatment, and sex were included as independent variables. In addition, we tested for effects of initial lizard mass, temperature, and sex on the length of time survived by lizards. Second, we conducted analyses to determine the cause of winter mortality of lizards. For these analyses, we conducted factorial analyses of covariance (ANCOVA) to determine if demise, temperature treatment, initial lizard mass, lizard sex or survival time significantly affected hydration or organ/whole-animal energetics. Specifically, we predict a significant difference related to demise for those traits (dependent variables) that act as vital (limiting) store for survival such that animals dying naturally will have less of that store than those sacrificed. Demise and temperature treatment were included as factors in these analyses, while initial lizard mass, lizard sex, and survival were included as covariates. Our dependent variables were mass loss, water content, liver mass, liver glycogen content, liver lipid content, liver energy content, fat-body mass, fat-body energy content, tail energy content, carcass energy content, and total energy content. For all ANCOVAs, we began by testing for homogeneity of regression coefficients to determine if ANCOVA was appropriate [p. 497 (Pedhazur, 1982)]. To do this, we utilized the R^2–change test [p. 62 (Pedhazur, 1982) to calculate the proportion of variance incremented by appropriate product vectors and determined statistical significance of resulting F-ratios using Microsoft Excel. However, due to the large number of dependent variables, we used a Bonferroni correction for these tests and only considered regression coefficients to be heterogeneous if $P<0.005$. Following this, we also determined if each covariate significantly affected the dependent variable prior to including that covariate in the final model (using effect tests). However, in this case we deemed an independent variable significant if $P<0.05$. Any non-significant independent variable/covariate ($P>0.05$) was removed from the final model. Finally, we tested for differences among treatment groups using effect tests reported by JMP. Again, we only report effects as significant if $P<0.005$. While we describe these procedures here, for brevity we only present the final model in our results. Third, for the winter hydration experiment, we conducted analyses on survival (logistic regression) and mass loss of survivors (ANOVA). In each case, we conducted a full-factorial analysis that included hydration treatment (yes/no), lizard sex, initial mass, and their interactions as factors.

RESULTS
Winter survival experiment

Of the 88 lizards that began the winter survival experiment, 26 died naturally over the course of the 149-day winter (41.9% mortality rate excluding animals that were sacrificed). Of these, eight were

Table 1. Mean (±1 s.e.m.) of response variables of *Uta stansburiana*, split by mode of death (demise), winter temperature treatment and lizard sex

Demise	Temperature	Sex	N	Mass loss (%)	Water content (%)	Liver mass (mg)	Liver glycogen (μmol g^{-1})*	Liver lipid (%)	Fat-body mass (mg)	Fat-body lipid (%)	Tail-base lipid (%)	Carcass lipid (%)
Natural	Cold	Male	4	7.7±3.56	70.4±0.71	91±27.6	91±30.4	2.6±0.87	11±2.9	22.8±12.73	2.6±0.96	13.0±0.62
Sacrificed	Cold	Male	4	4.8±0.46	73.1±0.92	80±19.4	119±29.3	10.3±3.95	20±8.1	76.3±37.57	3.9±2.37	12.9±0.73
Natural	Warm	Male	10	10.4±1.24	70.2±0.51	76±11.1	42±13.4	5.8±1.57	10±2.5	45.8±11.95	3.1±0.87	10.0±0.83
Sacrificed	Warm	Male	10	8.6±1.33	70.3±0.67	80±9.8	107±42.2	3.6±1.13	20±11.2	29.5±8.96	1.6±0.35	12.4±0.48
Natural	Cold	Female	4	8.8±0.62	70.3±1.31	52±7.8	21±5.9	10.4±3.93	8±2.8	73.0±30.15	4.4±1.88	16.3±1.31
Sacrificed	Cold	Female	4	6.8±1.28	71.6±0.44	55±6.9	127±81.4	8.1±5.24	6±2.2	53.7±12.08	7.1±2.36	12.1±0.76
Natural	Warm	Female	8	11.3±1.22	69.0±1.05	53±4.1	49±26.1	6.3±1.41	13±5.4	59.8±18.62	7.8±2.46	13.4±1.09
Sacrificed	Warm	Female	8	8.3±1.71	71.5±1.15	59±9.5	122±55.0	5.9±0.85	22±11.2	43.5±9.46	6.4±2.54	12.2±0.80

*Reported as μmol glucose units per g liver fresh mass.

from the cold treatment while 18 were from the warm treatment (Table 1). An additional 26 animals were sacrificed to compare to these natural deaths. The remaining 36 lizards survived the entire lab winter (149 days). A chi-square (χ^2) test indicated that animals were more likely to survive the experiment if they were in the cold treatment [cold survivors, 28 of 36 (77.8%); warm survivors, 8 of 26 (30.8%); χ^2=15.49, d.f.=1, $P<0.001$], were initially larger in body mass [survivors, 2.64±0.132 g; non-survivors, 2.32±0.199 g; χ^2=6.93, d.f.=1, P=0.009] and were female [female survivors, 20 of 32 (62.5%); male survivors, 16 of 30 (53.3%); χ^2=4.81, d.f.=1, P=0.028]. However, only temperature treatment was significantly related to the number of days survived (cold survival, 141.8±2.53 days; warm survival, 116.0±6.61 days; $F_{1,59}$=14.92, $P<0.001$). Duration of survival was not related to lizard sex ($P>0.05$) and only marginally related to initial mass ($F_{1,59}$=3.60, P=0.063).

Determination of the critical limiting (vital) store necessitated analyses on multiple dependent variables (Tables 1, 2). These analyses began with a test of variation among regression coefficients. For all independent variables, we detected no variation among treatment groups, indicating that ANCOVA was appropriate in all cases. For three dependent variables (size-relative liver mass, liver lipid content, relative liver total energy content), no independent variable or covariate was significant ($P>0.100$) and thus no further results are reported. For the remainder of the dependent variables, at least one independent variable (factor or covariate) significantly affected that trait, for which we report significant ($P<0.005$) or marginally significant ($P<0.05$) effects (Table 3). For percent mass loss, we found that temperature treatment (cold, 7.0±0.94%; warm, 9.9±0.68%) and

initial mass (slope, $-10.4x$) were significant (Fig. 2A), but the effects of demise (natural, 10.0±0.80%; sacrificed, 8.0±0.79%) and survival time (slope, $10.0x$) were only marginal. Water content (Fig. 2B) had only marginal effects on demise (natural, 69.9±0.43%; sacrificed, 71.3±0.48%) and initial mass. For liver glycogen content (Fig. 3), only demise was significant (natural, 48.4±10.55 μmol g^{-1} liver; sacrificed, 116.5±25.70 μmol g^{-1} liver). For size-relative fat-body mass, initial mass (slope, $1.40x$) and (marginally) sex (females, 0.008±0.0013 g; males, 0.005±0.0009 g) were significant. Likewise, initial mass (slope, $2.49x$) and (marginally) sex (females, 0.05±0.015 kcal death^{-1} mass; males, 0.03±0.009 kcal death^{-1} mass) were significantly related to size-relative fat-body energy content. Similarly, initial mass (slope, $1.94x$) and sex (females, 5.3±0.86%; males, 2.6±0.49%) were significantly related to tail lipid content. For percent carcass lipid content, only temperature treatment was marginally significant (cold, 3.4±0.17%; warm, 3.9±0.14%). Finally, for size-relative total energy content, only demise was marginally significant (natural, 4.0±0.20 kcal death^{-1} mass; sacrificed, 3.6±0.11 kcal death^{-1} mass).

Winter hydration experiment
When we tested for the effects of winter hydration on survival (yes/no), only initial mass was significant (slope, $9.6x$; χ^2=16.28, d.f.=1, $P<0.001$), with larger lizards tending to survive longer. Although the trend was toward greater survival due to hydration treatment, lizards that received misting did not differ in survival probability from those that did not [hydrated, 27 of 43 (62.8%) survived; non-hydrated, 22 of 42 (52.4%) survived; χ^2=2.01, d.f.=1, P=0.156]. For mass loss,

Table 2. Estimates (mean ±1 s.e.m.) of organ and total animal energy content (on per gram basis) of *Uta stansburiana*, split by mode of death (demise), winter temperature treatment and lizard sex

Demise	Temperature	Sex	N	Death mass (g)	Liver energy content (kcal g^{-1} at death)	Fat-body energy content (kcal g^{-1} at death)	Tail-base energy content (kcal g^{-1} tail)*	Carcass energy content (kcal g^{-1} wet carcass)[†]	Total lizard energy content (kcal g^{-1} at death)[‡]
Natural	Cold	Male	4	2.56±0.694	0.010±0.0020	0.022±0.0145	0.25±0.090	0.27±0.022	0.28±0.003
Sacrificed	Cold	Male	4	2.59±0.655	0.022±0.0025	0.034±0.0153	0.37±0.223	0.32±0.022	0.32±0.009
Natural	Warm	Male	10	2.53±0.352	0.019±0.0050	0.044±0.0252	0.29±0.082	0.36±0.020	0.40±0.047
Sacrificed	Warm	Male	10	2.50±0.288	0.017±0.0050	0.012±0.0038	0.15±0.033	0.34±0.016	0.33±0.017
Natural	Cold	Female	4	1.55±0.131	0.035±0.0142	0.017±0.0090	0.41±0.177	0.36±0.041	0.40±0.045
Sacrificed	Cold	Female	4	1.55±0.145	0.037±0.0151	0.029±0.0118	0.66±0.222	0.32±0.026	0.36±0.057
Natural	Warm	Female	8	1.63±0.149	0.017±0.0031	0.100±0.0387	0.55±0.166	0.45±0.036	0.54±0.081
Sacrificed	Warm	Female	8	1.78±0.241	0.021±0.0037	0.027±0.0097	0.39±0.126	0.33±0.025	0.31±0.023

*Because amount of tail sampled varied, reported as kcal per g tail sample.
[†]Carcasses lacked liver, fat bodies and tail, so data are reported as kcal per g remaining carcass.
[‡]Calculated by summing kcal in each category, then dividing by mass at time of death.

Table 3. Results (*F*-values) from factorial ANCOVAs used to determine causes of winter mortality of *Uta stansburiana*

	Mass loss (%)[1]	Water content (%)[2]	Liver glycogen (μmol g⁻¹)[3]	Fat-body mass (g g⁻¹ at death)[4]	Fat-body energy content (kcal g⁻¹ at death)[5]	Tail-base energy content (kcal g⁻¹ tail)[1]	Carcass energy content (kcal g⁻¹ wet carcass)[6]	Total lizard energy content (kcal g⁻¹ at death)[7]
Factors								
Demise	4.54*	5.17*	**8.59****					4.58*
Temperature	**13.21*****						5.42*	
Covariates								
Initial mass	**11.53*****	5.11*		**9.30****	**10.96****	**12.93*****		
Sex				5.89*	**8.31****	**12.57*****		
Survival time	6.58*							

Only independent variables with *P*-values <0.05 are included in final analyses. Due to the large number of comparisons, only independent variables with *P*<0.005 are considered significant and are shown in bold. *P<0.05; **P<0.01; ***P<0.001.
Degrees of freedom: ¹1,47; ²1,49; ³1,42; ⁴1,48; ⁵1,46; ⁶1,50; ⁷1,41.

only the effect of hydration was significant (hydrated, 0.10±0.011 g; non-hydrated, 0.18±0.014 g; $F_{1,29}$=14.28, *P*<0.001).

DISCUSSION
In this study, we set out to address the question: is winter survival in ectotherms related to energetics or hydration? We hypothesized that mortality occurs when some vital limit is reached and that the use of this limiting resource is temperature-dependent (Fig. 1). Thus, we expected a pattern of utilization in our winter survival experiment such that water and/or energy stores would slowly decrease to a critical point and that animals that died naturally would all have similar values in the critical store, but that still-living animals would have significantly higher values of that store at that point. Similar to previous research on this species (Zani, 2008), lizards in colder microenvironments survived the winter for longer periods of time and had a greater probability of surviving the entire length of our simulated winter (149 days). However, unlike that previous study

(Zani, 2008), initial body mass was not clearly related to survival duration in this experiment. The only other major difference related to treatment temperature in this experiment was related to mass loss, which was lower in animals experiencing a cold winter. Rather, the major findings of this study relate to mode of death: natural *vs* sacrificed.

In this study, we found that demise (natural *vs* sacrificed) was related to an individual's water content at death and liver glycogen content. When compared with animals that were sacrificed, animals dying naturally had both marginally greater mass loss and lower water content. Furthermore, sacrificed animals had significantly greater amounts of glycogen remaining in their livers than those that died naturally (Table 1). While fat-body size and the energetic content of fat bodies and tails differed between the sexes and in relation to initial body mass, these traits were not related to demise, indicating no difference between animals that died naturally and those still alive at the same point in time.
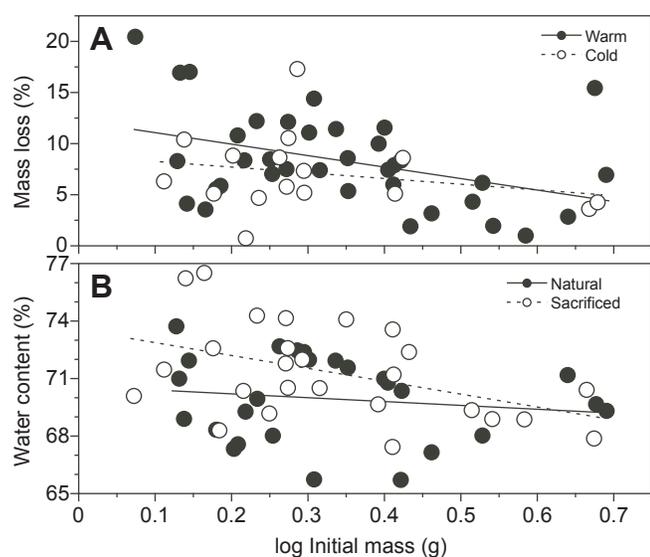

Fig. 2. Relationship between log initial lizard mass and (A) percent mass loss and (B) percent water content split by demise (natural/sacrificed). For mass loss, there was a significant relationship with initial lizard size (slope, −10.4*x*) as well as a difference between temperature treatments (cold, 7.0±0.94%; warm, 9.9±0.68%). For water content, there was a marginal relationship with initial lizard size (slope, −4.4*x*) as well as a marginal difference between modes of death (natural, 69.9±0.43%; sacrificed, 71.3±0.48%).
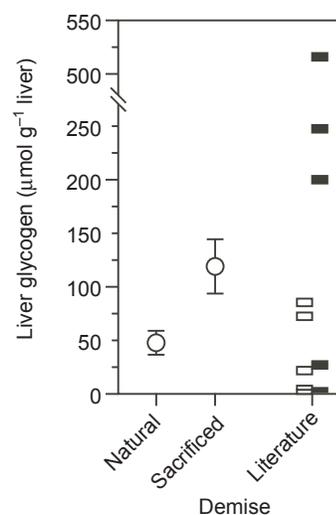

Fig. 3. Relationship between average (± s.e.m.) lizard liver glycogen content and demise (open circles), showing a significant final difference in glycogen stores (see also Fig. 1) between animals dying naturally and those sacrificed at the same time. Rectangles are reports from previous studies indicating the range of variation for different lizard species in liver glycogen during winter (open) and summer (filled) estimated from sacrificed animals (see Table 4).

While we cannot know just how close to their vital limits (i.e. mortality) animals in our sacrificed group were, at first glance these results suggest a role in winter mortality for both hydration and energetics related to glycogen storage. However, there are reasons to discount the importance of hydration for survival. The difference in water content between natural deaths (69.9±0.43%) and sacrificed animals (71.3±0.48%) is rather modest (only 1.4%). Furthermore, previous studies on the vital limits of water loss in lizards (e.g. Heatwole and Veron, 1977; Munsey, 1972; Sexton and Heatwole, 1968) all indicate a much greater tolerance to dehydration than observed here. For example, among the 15 species included in previous reports, the lowest percentage of mass that can be lost due to dehydration before mortality was 19% in a species that inhabits tropical rainforest (Heatwole and Veron, 1977). For western fence lizards (*Sceloporus occidentalis*), a desert species closely related to *U. stansburiana*, 39% of mass can be lost before death (Munsey, 1972). For our winter survival experiment, we found that mass loss increased with time but averaged 10.0±0.80% among animals that died naturally. However, 12 lizards (six male, six female) that were sacrificed by us just prior to the start of this experiment, but were otherwise maintained on identical experimental conditions, had water contents of 75.2±0.60%, which is only 6.3% greater than the water content at death of naturally dying animals (Table 1). These data suggest that mass loss due to actual water loss was less than 10% of initial body mass. However, the maximal possible water loss (assuming that all mass lost by lizards in our experiment was due to water loss and not metabolized energy stores) is only half the previously reported mass loss related to vital limits for any lizard species (see above). As a further test of the idea that hydration is not related to winter survival, we conducted a recovery experiment in which we hydrated (misted) lizards during lab-imposed winter conditions. For this experiment (see 'Winter hydration experiment' in Results), we hypothesized that if water is limiting, the presence of moisture should minimize water loss as well as increase survival. While lizards receiving no hydration during winter lost nearly twice as much mass (0.08 g), they did not survive any better than those that were hydrated. Taken together, we conclude that the vital limit of lizards during our winter-survival experiment was not related to hydration. Rather, our results implicate some aspect of energetics as the critical limit for winter survival.

As it pertains to overwintering energetics, we found no evidence that lipid stores were being utilized during exposure to winter. That is, lipid in the liver, fat bodies and tail were all unrelated to either the length of winter or the mode of death of the individuals (Table 1). Thus, it does not appear that lipids are the critical limiting factor for survival or even that total energy content of animals was limiting. Rather, only one aspect of overwintering energetics was related to demise: animals that died naturally had lower levels of liver glycogen (Fig. 3; Table 3). Specifically, the glycogen content of animals that died naturally (48.4±10.55 μmol g^{-1} liver) was less than

half that of animals still alive at the same time point (116.5±25.70 μmol g^{-1} liver). Therefore, our results indicate that only glycogen stores in the liver appear to be of energetic importance during winter. Several previous studies have also reported that liver glycogen levels decrease substantially during winter (e.g. Dessauer, 1955; Barwick and Bryant, 1966; Haggag et al., 1966; Patterson et al., 1978; Taylor, 1986; de Souza et al., 2004). For comparison with our results, we used data reported in these previous studies (where available) to estimate liver glycogen in both winter and summer in equivalent units (Table 4). Based on this, we report that previous quantification of liver glycogen in hibernating lizards varied somewhat, but averaged ~40 μmol g^{-1} liver in the winter and ~200 μmol g^{-1} liver in the summer (Table 4; Fig. 3). These previous studies were also based on animals kept in lab conditions during winter, but all animals were presumably still alive at the time of assay so the actual vital limit is not clear. However, the glycogen levels in our sacrificed group (116.5±25.70 μmol g^{-1} liver), which is the most relevant group for comparison, falls between the winter and summer values of most previous studies (Fig. 3). Indeed, it is only due to our novel experimental design (Fig. 1), in which we assayed animals still alive as well as those that died naturally, that allows us to report for the first time a vital limit for glycogen of overwintering lizards.

Why is overwintering survival apparently not related to lipid stores? One cause of this pattern may be related to cessation of energy metabolism at low temperatures. For example, animals may experience cold-induced reduction of mitochondrial enzyme activity in winter (Joanisse and Storey, 1994; McMullen and Storey, 2008) or even mitochondrial degradation (Kukal et al., 1989). This may make lipid metabolism at low temperatures problematic. However, at least one study of mammals indicates the opposite; that animals are highly lipolytic during overwintering (Florant et al., 1993). A second possible cause of observed patterns is that lizards are utilizing only certain types of fatty acids. For example, marmots lacking essential fatty acids aroused more frequently and utilized more energy to survive winter (Florant et al., 1993). In mammals at least, polyunsaturated fatty acids appear related to longer hibernation and lower metabolic rates during hibernation (Geiser, 1990; Geiser and Kenagy, 1987). Thus, it may not be absolute amounts of lipid stores that are important but composition of those stores. However, we know of no studies that have reported the fatty acid composition or patterns of utilization of lizards during winter (but see Simandle et al., 2001; McCue, 2008). A third possibility is that lizards sequester lipids primarily for subsequent (i.e. during spring) reproduction. Although numerous studies, including a study of side-blotched lizards (Hahn and Tinkle, 1965), have reported that fat-body size decreases during winter (e.g. Telford, 1970; Guillette and Sullivan, 1985; Naya et al., 2008) (for a review, see Derickson, 1976), presumably as lipids are metabolized, other studies have indicated little or no such seasonal change (e.g. Afroz et al., 1971), or rapid

Table 4. Liver glycogen estimated from published reports in the literature

Species	Winter glycogen (μmol g^{-1} liver)	Summer glycogen (μmol g^{-1} liver)	Source
Anolis carolinensis	6.9	26.7	Dessauer, 1955
Egernia cunninghami	2.0	4.4	Barwick and Bryant, 1966
Varanus greseus	86.3	246.2	Haggag et al., 1966
Lacerta vivipara	22.2	516.2	Patterson et al., 1978
Tupinambis merianae	73.3	200.9	de Souza et al., 2004
Uta stansburiana	116.5		Present study

Winter is defined as during or immediately following winter; summer is defined as during summer or fall (i.e. immediately prior to winter).

decrease of lipid stores only following emergence in the spring (Etheridge et al., 1986; Goldberg, 1972). It is important to note that such studies are often conducted by sampling different individuals at each time point and not the same individuals repeatedly, which would be difficult, if not impossible, because of the destructive nature of sampling. However, this means that actual changes in individuals are difficult to quantify, which is why we utilized our particular experimental design (Fig. 1). Yet, the lipids contained in the fat bodies of side-blotched lizards are utilized for reproduction in the spring, and removal of these energy stores delays reproduction by the time it takes to replace them (Hahn and Tinkle, 1965). Thus, we propose an alternative interpretation of previous studies. We suggest that certain lipid stores, such as abdominal fat bodies, may not be readily available for utilization during winter due to the temperature-dependent physiological limitations related to mobilization. Rather, these energy stores (i.e. abdominal fat bodies) may be utilized immediately following winter to enable survival and/or advance reproductive phenology at the beginning of the growing season. Clearly, this area needs further research to disentangle these issues.

Climate change, winter temperatures and population persistence

Recent research on insects and lizards (Pullin and Bale, 1989a; Pullin and Bale, 1989b; Irwin and Lee, 2000; Irwin and Lee, 2003; Williams et al., 2003; Zani, 2008) has suggested that milder winters may negatively impact ectotherm populations due to the relationship between temperature and metabolism. Colder winters appear to increase energy conservation (Pullin and Bale, 1989b), winter survival (Pullin and Bale, 1989a; Irwin and Lee, 2000; Wilson and Cooke, 2004; Zani, 2008) and subsequent fecundity (Irwin and Lee, 2000; Williams et al., 2003). Together, these previous findings strongly implicate energetics as a limiting factor in overwinter survival for ectotherms. However, the assumption that ectotherm mortality is caused by a lack of energy *vs* some other critical limit has not been explored before now. The results of the present study extend our understanding of overwintering physiology by suggesting that survival of side-blotched lizards during winter is related primarily to energetics, namely glycogen stores in the liver. That is, our results are consistent with previous research but, rather than implicate lipid stores as vital, they shift the focus to carbohydrate stores in the liver.

Observations of the patterns of temperature change in recent decades have revealed that winters are becoming milder (DeGaetano, 1996; Easterling et al., 1997; Easterling et al., 2000). Thus, warmer winters have the potential to increase individual mortality unless lizards are capable of behaviorally modifying their location within the hibernacula. However, growing-season lengths are also being altered by climate change (Schwartz and Reiter, 2000; Bradshaw and Holzapfel, 2001; Beaumont et al., 2006; Schwartz et al., 2006), which should shorten the length of the unfavorable season. As with other ectotherms, side-blotched lizard body size within a population is positively related to length of growing season (Zani, 2008), which suggests any detriment due to milder winters could be offset by changes in the growing season. Lizards at this population in eastern Oregon must withstand an inactive period of nearly 150 days and are near their latitudinal and altitudinal limits (Nussbaum et al., 1983; P.A.Z., personal observation), which are possibly imposed by the physiological limitations of individuals. Our findings suggest a mechanism for the limits of population persistence and, by extension, the limits of this species' geographic distribution. More generally, populations (or species) may not be limited by growing-season length but rather by physiological tolerances associated with the length of the inactive season (i.e. winter length). If true, climate change may actually be making winter less of a limit to species distributions (by shortening winter) but may impose more physiological hardships to individuals (higher rates of dehydration and metabolism) that must be offset by growth and storage during the favorable season. While physiological (particularly thermal) limits to species' ranges are of increasing research importance, previous work has typically focused on limits imposed (or removed) during the growing season (for reviews, see Porter and Tracy, 1983; Pörtner, 2002; Helmuth et al., 2005) (but see also Ultsch, 1989). Thus, we advocate for increased attention to physiological limits imposed during the unfavorable season as well as to the ongoing alteration of those limits by anthropogenic climate change.

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