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ANIMAL SCIENCE

Wintertime tales: How the lizard *Liolaemus lineomaculatus* endures the temperate cold climate of Patagonia, Argentina

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Abstract: In temperate, polar and montane environments, ectotherms must find ways to endure throughout the coldest months of the year. Lizards search for microsites where temperatures remain warm or alter their biochemical balance to tolerate freezing or avoid it by supercooling. We evaluated the cold hardiness and potential winter refuges of two populations of *Liolaemus lineomaculatus*, from a temperate site (42°S) and a cold site (50°S). We analysed the role of possible cryoprotectants by comparing a group of cooled-down lizards with a control group of lizards that were not exposed to cold. The populations of this study are not freeze tolerant and the biochemical analysis showed no evidence of metabolites significantly changing concentration after exposure to cold. However, the species remained several hours at their Supercooling Point (SCP), suggesting they can supercool. The analysis of potential winter refuges showed that lizards using these potential refuges would spend almost no time at all at temperatures close to or below their SCP. Furthermore, lizards from the cold site were able to survive below 0°C temperatures with a lower SCP than lizards from the temperate site. *Liolaemus lineomaculatus* developed physiological mechanisms that can help them survive when temperatures drop sharply, even when lizards are in suitable shelters.

Key words: Cold hardiness, cryoprotectants, *Liolaemus lineomaculatus*, Patagonia, supercooling point, winter refuges.

INTRODUCTION

In temperate and cold habitats, ectotherms such as lizards must spend at least half of their lives coping with the challenges related to sub-zero environmental temperatures and stressors associated with overwintering (Williams et al. 2015). Even when environmental temperatures are above 0°C, cold weather can still have a negative effect on activity thresholds. Temperatures below the Critical Thermal Minimum (CT_{Min}) (*sensu Cowles & Bogert 1944)* render the animals unable to escape predators (Christian & Tracy 1981) or forage to obtain resources for overwintering, changing the dynamics of energy storage (Tattersall et al. 2012).

Furthermore, in these harsh environments, temperatures frequently reach negative values and, when behavioural options (such as burrowing) are insufficient, lizards can respond by adopting one of two physiological mechanisms: freeze tolerance or freeze avoidance by supercooling. Freeze tolerance is a mechanism where the lizard tolerates the partial conversion of body fluids into ice for a variable amount of time, with high variation among species and populations in the resistance to a different percentage of frozen body fluids, time frozen, and the number of

freezing and thawing episodes individuals can tolerate (Voituron et al. 2002, Berman et al. 2016). Meanwhile, by supercooling, the individual "can remain unfrozen at temperatures below the equilibrium crystallization temperature of its body fluids" (Costanzo et al. 1995). This mechanism involves less physiological stress (Costanzo et al. 2008), but there is a risk of spontaneous freezing at temperatures below the equilibrium freezing point (Salt 1966), with potentially lethal consequences (Storey & Storey 1996). Despite their differences, both freeze tolerance and freeze avoidance require a stable temperature to improve the chances of survival of the overwintering individuals (Pauli et al. 2013). Moreover, these mechanisms involve biochemical variables such as urea, glucose, and lactate changing concentrations and increasing osmolality (Costanzo et al. 2000, Grenot et al. 2000, Voituron et al. 2002), and the synthesis of Anti-Freeze Proteins (AFPs), that help to avoid freezing and recrystallization in supercooling and freeze tolerance, respectively (Storey & Storey 1986).

Populations of the same species living in environments with different climates may develop different cold hardiness capabilities, even if the geographical separation (such as in latitude or elevation) is not large; however, there is not a clear pattern or correlation. For example, the CT_{Min} of a South American gecko (*Homonota darwinii*) showed changes among populations in correlation with cooler climates, although no pattern was found regarding latitude (Weeks & Espinoza 2013). Additionally, some studies show an effect of latitude or elevation in cold hardiness parameters of terrestrial ectotherms such as CT_{Min} (Sunday et al. 2011, Munoz et al. 2014, Huang et al. 2006, Winne & Keck 2005). However, there are also studies showing interpopulation differences in cold hardiness capabilities that could not be explained by winter severity (Michels-Boyce & Zani 2015), differences that are better explained by other factors (Voituron et al. 2004, Costanzo et al. 2006, 2004, Spellerberg 1972), or even no interpopulation differences at all (Gvozdík & Castilla 2001, Yang et al. 2008). Nevertheless, climatic differences at the landscape scale among populations may not be representing accurately what lizards experience in the microsite where they choose to spend the winter.

Microsite selection is of paramount importance in winter survival. Overwintering animals heavily rely on thermally stable structures that protect them from predators, extreme weather variations, and other disturbances (Williams et al. 2015, Kinlaw 1999, Huey 1991). Furthermore, refuge availability can have a larger impact on overwintering than the thermal quality of the habitat as a whole (Monasterio et al. 2009). A recent potential refuge analysis showed that choosing appropriate refuges might allow the lizard *Liolaemus pictus* in the high elevation forest in the north of Patagonia, Argentina, to endure the cold environmental conditions without resorting to physiological mechanisms such as freeze tolerance or supercooling (Cecchetto et al. 2019). Thus, unless lizards find a suitable winter refuge, they would experience sub-zero environmental temperatures during extended periods in the steppes at the highlands and high latitudes of Patagonia, Argentina, under a snowpack that reaches a considerable depth (>1m).

In this study, we analysed the cold hardiness by physiological and behavioural mechanisms of a lizard, *Liolaemus lineomaculatus* (Liolaemidae), a viviparous species with a broad distribution from the high Andes in the north-west of Patagonia, in Neuquén province (39° S), at elevations up to 1800 m asl, to the lowlands in Santa Cruz province (400 m asl 51° S; Cei 1988, Scolaro 2005).

We propose that *L. lineomaculatus*, living at higher latitude and elevation than *L. pictus*, must have developed a cold hardiness mechanism such as supercooling or freeze tolerance to survive the coldest months of the year in the steppes near the cities of Calafate and Esquel. Additionally, we predict that after experimental exposition to cold, individuals of *L. lineomaculatus* will show a significant increase in the concentration of at least one of the selected biochemical variables (urea, total proteins, glucose, lactate), previously identified as cryoprotectants in other lizard species (Costanzo et al. 2000, Grenot et al. 2000, Voituron et al. 2002). Moreover, we also expect to find differences between *L. lineomaculatus* populations in the minimum temperatures experienced throughout the year and in the amounts of hours at sub-zero temperatures within potential winter refuges or at surface level. Furthermore, we hypothesize that these two populations must have diverged in their cold-hardiness capacities, varying with the temperatures of the environment and thermal quality of available refuges. From this hypothesis, we predict that the *L. lineomaculatus* population in the colder environment (Calafate) will show a lower CT_{Min}, a lower supercooling point, or both, than the population located in the milder environment in Esquel.

Studies that integrate the physiological, behavioural, and ecological responses related to winter survival with the availability of potential overwintering microsites in populations located at different latitudes and elevations are relevant to understand underlying processes of cold hardiness, especially given the lack of studies on this subject for species in the Southern Hemisphere. While our previous work (Cecchetto et al. 2019) focused on a single population of *L. pictus* that showed mild cold hardiness, in this study, we evaluate intraspecific differences in the restraints and opportunities for two populations located at the latitudinal and altitudinal extreme of the distribution of one of the southernmost species of Patagonian lizards.

MATERIALS AND METHODS

Study areas and capture methods

We captured adult males of *L. lineomaculatus* in the steppes of Calafate, the cold site (50°15´ S, 71°29´ W; 450 m asl; February 2018, N= 20), and on a mountain in Esquel, the temperate site (42° 49´ S, 71° 15´ W; 1800 m asl; March 2019; N=19), in Argentina. Captures were made between the end of summer and the beginning of autumn considering that, at the selected locations, it is a period of the year when air temperatures can rapidly change and result in temperatures that are close or below CT_{Min} (Supplementary Material - Figure S1). In addition, the carbohydrates used as cryoprotectants by terrestrial animals are synthesized almost exclusively from reserves obtained during late summer and early autumn feeding (Storey 1997). Therefore, captures were made in the limit of the brumation of *L. lineomaculatus*, which in the steppes at high latitudes and elevations starts in mid-autumn (May), and lasts until spring (September; Medina et al. 2011).

In the steppes of Calafate, the typical terrain is a plain, open field with frequent bushes and tussocks, but almost no boulders or rocks for lizards to hide under. In the high-Andean steppes of Esquel, *L. lineomaculatus* can find refuge under boulders, bushes, tussocks or in the many abandoned burrows of small mammals (such as rodents from the genus *Ctenomys*). In a recent study we found that in Esquel, lizards spent the majority (95%) of their hours of activity in autumn, spring and the beginning of summer within their thermal tolerance breadth (*i.e.*, at temperatures between their CT_{Min} and

their CT_{Max}), while in Calafate, during the same months, lizards spent only 71% within their thermal tolerance breadth (Cecchetto et al. 2020). Lizards were captured by hand or loop, and we measured body temperature (Tb) immediately after capture, using a digital thermometer (± 0.1°C; Omega 871A, type K 9 thermocouple; Stanford, CT) connected to a catheter probe introduced about 1 cm inside the cloaca. We handled individuals by the head and hips within 10 seconds of capture to avoid heat transfer.

Potential lizard refuges

To understand the challenges that the highlands and tablelands of Patagonia represent for the studied populations in the potential refuges lizards use during the colder months, we placed four lizard models in Calafate and six lizard models in Esquel with thermistors, connected to data loggers (HOBO TEMP® H8, four-channel external data logger and its thermistors) between March 2017 and January 2018. Temperature values were recorded for these 11 months every 30 minutes. The models were made of PVC pipes (1.5 cm diameter × 8 cm length) which were then sealed at the ends with silicone (Fastix®) and painted grey to mimic body size, reflectance, thermodynamics, and shape of lizard's bodies. To determine if the model was a good indicator of the temperature that a non-thermoregulatory lizard would attain in the environment or if corrections were needed, we performed simultaneous trials for calibration in two identical terraria using the PVC model in one terrarium and a live lizard on the other. During the trials, we moved the PVC model to mimic the movements of the live lizard. Subsequently, we regressed model temperature on lizard body temperatures (T_b= 2.82 + 0.912 × Physical model. Regression: Adjusted R^2= 0.92; n= 2510; Confidence Interval= 0.88 - 0.94), and amended the values accordingly.

Following the calibration, we placed two models (one at each location) on the ground, partially covered but exposed to environmental temperatures, as a reference point representing temperatures typically experienced just outside any type of refuge. We then selected the potential refuges in which the species might seek temporary shelter, to include the variety of microenvironments at both sites (*e.g.*, buried ~10-15 cm underground; beneath rocks; under tussocks) and placed additional PVC models in the potential refuges. These potential refuges are speculative predictions of where lizards may choose to spend the winter, based on what they had available in the environment and burrows used by them during activity season (from September to March, when not in brumation, personal observation). The natural history information in the literature of species of similar size with similar thermal ecology (*Zootoca vivipara,* under a boulder -Fellenberg 1983-; or buried 5-15 cm in the ground / under vegetation -Berman et al. 2016-) was also considered. For the potential refuges, we recorded the number of periods or events when the temperature dropped below 0°C and the duration of each period (*i.e.,* time until temperature raised again above 0°C). In this way, if a potential refuge spent 5 hours above 0°C, 2 hours at negative temperature values and 3 hours later above 0°C, this would be registered as a single period below 0°C that lasted for 2 hours.

In addition, to compare the "thermal quality" of potential refuges, we applied the concept of degree-days (*sensu Lindsey & Newman 1956)*, using as reference the value of 0°C (the melting point of water at 1.01325 x 10^5 Pa). Degree-days are the summation of temperature differences to a reference value over time. In this way, degree-days explain both the magnitude and duration that lizards would experience temperatures in relation to a reference chosen value. This

metric allows a direct comparison of thermal regimes among different sites for many species or species populations (Guisan & Hofer 2003, Schwanz & Janzen 2008, Murphy et al. 2010, Boyero et al. 2011, Graae et al. 2012, Mitchell et al. 2012).

This reference value of 0°C allows inferences about how much and for how long *L. lineomaculatus* (that overwintered in the selected potential refuges or in no shelter; *i.e.*, surface-level model) would be subjected to temperatures below the melting point of water. We used degree-days to compare potential refuges, and it was calculated as:

$$HRDD\,0 = \sum_{i=1}^{n} \left| (Ti - 0)/24 \right|$$

where HRDD0 is heating refuge degree-day for 0°C, and T*i* refers to registered temperature values below 0°C (every 30 minutes).

Laboratory experiments and housing conditions

We brought the lizards ($N_{Calafate}$ = 25 + N_{Esquel} = 25) to the laboratory where we measured snout-vent length (SVL) and body mass (Table I) using a digital calliper (± 0.02 mm) and an Ohaus balance Scot Pro (± 0.01 g), respectively. Lizards

Table I. Descriptive data (Mean ± *SD*) of Scaled Mass Index (SMI) and critical minimum temperature (CT_{Min}, °C) of *Liolaemus lineomaculatus* from Calafate and Esquel. The hyphen symbol in CT_{Min} (-), corresponds to absent data (control individuals).

Calafate		Esquel	
CT_{Min} (°C)	Scaled Mass Index	CT_{Min} (°C)	Scaled Mass Index
-	4.75	-	3.93
-	4.66	-	3.87
-	4.85	-	3.78
-	5.74	-	3.83
-	4.74	-	3.49
-	4.29	4.75	4.32
-	4.93	4.54	3.39
-	4.40	6.21	3.61
-	4.83	5.22	4.32
-	4.75	5.44	4.41
5.79	4.08	5.87	4.61
4.86	5.21	4.19	4.28
2.23	4.62	5.27	3.82
4.17	5.61	5.58	4.05
3.81	4.55	5.19	3.00
5.86	4.66	5.11	3.86
5.36	4.93	3.97	5.59
2.77	5.16	4.83	3.90
3.09	5.11	5.59	3.77
5.01	4.79	4.81	3.68
Means (± SD)		Means (± SD)	
4.30 (1.29)	4.83 (0.4)	5.10 (0.61)	3.98 (0.54)

The hyphen symbol in CT_{Min} (-), correspond to control individuals.

were brought to the laboratory in individual cloth bags to minimize stress and were housed in individual open-top terraria (15 × 20 × 20 cm) at room temperature (day maximum ~20°C, night minimum ~10°C) for a maximum of 48 hours before the experiments, with a photophase of approximately 12 hours. Within these terraria, lizards were supplied with a refuge (a cardboard cylinder ~10 cm length x 5 cm diameter). Lizards were provided with water *ad libitum,* except for 5 hours just prior to the experiments, to avoid getting moisture on their body, which could freeze at negative temperatures, risking unwanted ice inoculation and freezing (to further avoid this situation, we manually blotted their skin dry with paper towels).

Supercooling point (SCP) determination

SCP was determined to evaluate if lizards from these populations are freeze tolerant. Additionally, this experiment also provided the supercooling point, understood as the lowest temperature before a peak, indicating the release of the latent heat of crystallization (Costanzo et al. 2008).

Two small subsets of animals, one for each population, were selected ($N_{Calafate}$= 5; N_{Esquel}= 6), and SCPs were determined. We placed lizards individually in dry plastic containers, positioned them in a freezer at 18°C for 30 minutes (until thermal stability was reached). We connected the lizards to a TC-08 Data Acquisition Module Omegas (8-Channel USB Thermocouple, ± 0.01°C) by ultra-thin (1 mm) catheter thermocouples, to register the body temperature and identify the exothermic reaction of body-water freezing. These thermocouples were fixed on the abdomen and not inside the cloaca since thermocouples placed in the cloaca at temperatures below 0°C can initiate unwanted freezing (Costanzo et al. 2008). SCP determination consisted of four stages. 1) lizards were cooled from 18°C to 0°C at a stable rate of -0.5°C*hour^{-1} for 36 hours. 2) lizards continued cooling at a rate of -0.25°C*hour^{-1} until an exothermic reaction was reached (*i.e.*, all lizards underwent crystallization of body water). 3) the lowest temperature (*i.e.*, the temperature of the last freezing exotherm) was maintained for 12 hours to ensure full body freezing. 4) finally, lizards were slowly thawed at a rate of 2.5°C*hour^{-1} until they reached at least the population's CT_{Min} and were taken out of the freezer and their vital signs (*i.e.,* breathing, movement, righting response) were checked.

For these experiments and the following cooling experiments, we used the lowest possible rates in relation to what a lizard would likely experience in the field (data from the lizard PVC models) to minimize any harmful effect on tissues from cooling too fast, but avoiding rates slower than actual rates experienced in refugia.

Biochemical cooling experiments

The cooled-down group ($N_{Calafate}$= 10; N_{Esquel}= 10) was placed individually in dry plastic containers positioned in a freezer, while a control group ($N_{Calafate}$= 10; N_{Esquel}= 9) was placed simultaneously in the same conditions at room temperature (20°C). Temperatures of the cooled-down group were regulated by a control in the freezer that allowed setting specific cooling rates and times. We connected the lizards to a TC-08 Data Acquisition Module Omegas (8-Channel USB Thermocouple, ± 0.01°C) by ultra-thin (1 mm) catheter thermocouples, to register the body temperature in both groups during the cooling experiment. A PVC pipe lizard model was set in another plastic container and exposed to the same temperature fluctuations as the lizards from the cooled-down group.

We performed the experiment in three stages, considering that lizards are normally exposed to air temperature fluctuations with smooth drops and extended periods hovering

near 0°C in this season, as seen in *Zootoca vivipara* (Grenot et al. 2000, Costanzo et al. 2008). In the first stage (From 20°C to 0°C), we exposed individuals to cold from the experimental starting temperature (20°C) to 0°C for 6 hours, at a rate of -3°C*hour^{-1}. In the second stage (Overnight), lizards stayed at approximately 0°C for 12h. Finally, in the third stage (Below 0°C), we dropped the freezer's temperature at a rate of approximately -0.75°C*hour^{-1} to ~-8°C for the Calafate individuals and to ~-6°C for the Esquel individuals. The final values for this stage were at first chosen by observing the lowest value obtained for each population in the analysis of the environmental temperatures. However, since values were below both populations' SCP and would have frozen lizards (and, given that they were not able to survive freezing, most likely killed them), we selected the closest value to the SCP that the freezer could achieve (-8°C and -6°C, respectively) and then maintained that temperature for 6 hours. Lizards were then warmed slowly at room temperature and examined for biochemical changes.

When computing cooling rates for analysis, in addition to raw temperature values, we used an Adjusted Body Temperature (AT_b) index to standardize the temperature change, considering that initial temperature values slightly varied among individuals. This index illustrates the temperature change independently from initial values as follows:

$$AT_b = ((T_b - T_{bi})/T_{bi}) * 100$$

where T_b is the body temperature at a given time, and T_{bi} is the body temperature at the beginning of the experiment.

At the beginning of the experiments, we monitored lizards to determine the critical minimum temperature (CT_{Min}; Table I), defined as the temperature at the lower extreme of tolerance at which the animal cannot right itself when placed on its back (*i.e.*, the loss of righting response *sensu* Doughty 1994). We evaluated CT_{Min} by quickly taking lizards out of the containers and placing them on their back as soon as they started reaching values of ~10°C. If the animal was able to right itself, it was placed back to continue cooling and the process was repeated every 30-60 seconds or every degree below the previous value, whichever happened first until the CT_{Min} value was found. To control for a potential effect of the handling on the individuals, such as a release of glucose caused by a sympathetic response, we handled all individuals in the control group in the same way as those in the treatment group.

At the end of the experiments, immediately after the extraction of individuals from the containers, we sacrificed lizards by decapitation, and then, liver and heart samples of each individual were individually stored in Eppendorf tubes and kept in a freezer until they were analysed the next day.

Milder complementary experiment for the temperate site individuals

Lizards from the cooled-down group from the temperate site, Esquel, were found dead after the experiment, presumably from cold exposure and not being able to supercool. Therefore, the control group was divided in two ($N_{cooled\ down}$= 6; $N_{control}$= 3) and the experiment was repeated using the same protocols but in the final stage (Below 0°C) we used a value 0.5°C higher than the lowest SCP detected for this population (-4°C, since the freezer's controller panel, didn't allow for non-integer values) and the temperature was maintained for less time (3 hours instead of 6), to ensure the survival of the individuals (Table SII). It should be noted that, given that these individuals were controls for the previous experiment, they underwent sub-zero temperatures only once.

Biochemical analysis

We analysed a liver and a heart sample per individual. We homogenized each sample (liver and heart separately) manually with a mortar, diluted it with physiological saline (9% V/V) in a 1:4 dilution, and then placed all samples in Eppendorf tubes to be centrifuged at 3200 rpm for 10 min. The material in each tube underwent absorption spectroscopy with enzymatic assays, to detect: urea, total proteins, and albumin. We adapted the methodology implemented in this study and made the selection of the cryoprotectants analysed considering biochemical variables found relevant in previous studies of cold hardening in reptiles (Costanzo et al. 2000, Grenot et al. 2000, Voituron et al. 2002); for urea, glucose, and Anti-Freeze Proteins (AFPs), respectively and in other taxa (Storey & Storey 1986; for AFPs). We inferred the presence of antifreeze proteins considering the differences between total proteins and albumin in the homogenate taking into account that an increase in total proteins without a corresponding increase in albumin would point to proteins related to the cooling experiment (although not necessarily AFPs). We determined all parameters for the supernatant using a Shimadzu UV-1800 spectrometer (Shimadzu Inc., Kyoto, Japan) with an absorption spectroscopy test with enzymatic assays and chemical reagents (Wiener Lab, Rosario, Argentina). The kits used were kinetic urea UV AA, total proteins AA and albumin AA. We previously reprogrammed the biochemical kits methods in relation to proportions and calibration values, to include sample values into the standard calibration curve and to obtain reliable results.

Given the lack of evidence for significant changes in the selected biochemical components after the experiment from Calafate and from a previous experiment with *Liolaemus pictus* (Cecchetto et al. 2019), analyses for Esquel individuals were focused only on glucose and lactate, which could be obtained from a drop of blood only.

Blood glucose and lactate

We measured glucose by taking a drop of blood from the caudal vein near the cloaca, avoiding the hemipenes, before and after the experiment, using a glucometer (Accu-Chek® Performa Nano, with a range of 10 mg/dL - 600 mg/dL) following the methodology of Voituron et al. (2002).

We calculated the proportional change in glucose or adjusted glucose change (ΔAGluc) to account for the difference in glucose initial values, given their uneven diet coming from the field, using the following formula:

$$\Delta AGluc = ((Gluc_f - Gluc_i)/Gluc_i) * 100$$

Where $Gluc_f$ was the glucose at the end of the experiment and $Gluc_i$ was the glucose at the beginning. Initial and final glucose were not analysed separately because the change in glucose was already analysed as ΔAGluc. ΔAGluc analyses the difference between initial and final glucose, accounting for individual differences in initial values, which is why we found ΔAGluc as a more relevant variable for this study.

We measured lactate by using another drop of blood from the caudal vein near the cloaca (from the same puncture made for the glucose measurement), before and after the experiment, using a lactometer (Lactate Scout+, SensLab GmbH, Germany, with a range of 0.5 - 25.0 mM). The small volumes of blood that we could obtain from lizards without harming the animals limited us to only one measurement on each individual, one for glucose and one for lactate.

We also calculated the proportional change for lactate, or adjusted lactate change (ΔALac) to account for the difference in lactate initial values, using the following formula:

$$\Delta ALac = ((Lac_f - Lac_i)/Lac_i) * 100$$

Where Lac_f was the lactate at the end of the experiment and Lac_i at the beginning. Initial and final lactate were not analysed separately because the change in lactate was already analysed as ΔALac (in the same way as ΔAGluc).

Statistical analyses

We made comparisons of glucose (ΔAGluc) and lactate (ΔALac) for each individual before and after the cooling experiment using a paired *t*-test, and comparisons for urea, total proteins, and albumin using ANCOVA between control and experimental groups, with the scaled mass index (SMI) as a covariable. Comparisons among potential refuges in degree-days were performed with a χ^2 test. In the case of Esquel, where multiple measures were taken for control individuals of the first experiment, a mixed model was performed with the '*lme4*' package for R (Bates et al. 2015) to compare ΔAGluc and ΔALac.

We analysed the variability in body sizes and weights using scaled mass index (SMI), calculated as:

$$SMI = M_i * [\ \langle\!\langle SVL_0 \rangle\!\rangle\ /\ \langle\!\langle SVL_i \rangle\!\rangle\ ]^{b_{SMA}}$$

Where M_i and SVL_i are the mass and SVL of the individual, SVL_0 is the arithmetic mean SVL of the population, and b_{SMA} is the standardized major axis slope from the regression of ln body mass on ln SVL for the population (*sensu Peig & Green 2009)*. The b_{SMA} exponent was calculated using the package '*lmodel2*' (Legendre 2014) in R (R Core Team 2019). All the other analyses were performed using the same software, with the '*nlme*' (Pinheiro et al. 2017) and '*car*' (Fox & Weisberg 2011) packages. The significance threshold for p values was set at 0.05.

Captures were carried out with authorization from the Wild Life Service of the Province of Chubut (Permit # 0460/16 MP; Disposition # 11/2016). We followed the ASIH/HL/SSAR Guidelines for Use of Live Amphibians and Reptiles as well as the regulations detailed in Argentinean National Law #14346.

RESULTS

Body size (SVL), weight, and scaled mass index (SMI)

Body size and body mass ranged from 50.13 to 60.74 mm and from 3.67 to 6.57 g for Calafate's individuals and ranged from 41.79 to 57.19 mm and from 3.01 to 5.66 g for Esquel's individuals. There were no significant differences in the SMI between control (mean= 4.80 ± 0.39) and cooled-down individuals (mean= 4.87 ± 0.427) from Calafate (ANOVA: $F_{1;\ 18}$= 0.183; p= 0.674) or between control (mean= 4.03 ± 0.31) and cooled-down individuals (mean= 3.92 ± 0.70) from Esquel (ANOVA: $F_{1;\ 17}$= 0.054; p= 0.818).

Field body temperatures, thermal microenvironments, and environmental temperatures in the field (degree-days)

Field body temperature of lizards was similar between sites (Table SIII). The exposed lizard PVC model (out of potential refuges) in Calafate reached a minimum value of -8.91°C, while the lowest temperature registered by lizard models in potential refuges was -3.37°C (Figure S1). In Esquel, the exposed model reached a minimum value of -8.70°C, while the lowest temperature registered by lizard models in potential refuges was -6.58°C.

The PVC lizard models in potential refuges in Calafate underwent 7 to 194 periods when they registered consecutive temperatures below 0°C that lasted between 1 and 3427 hours. Meanwhile, in Esquel, lizard models underwent between 7 and 69 periods of temperatures below 0°C that lasted between 1 and 103 hours (Table II).

Table II. Comparison between data obtained from ten lizard models set in potential locations of *Liolaemus lineomaculatus* for overwintering in Calafate and Esquel. Minimum, mean ± standard deviation (SD) and maximum number of consecutive hours with temperatures below 0°C in a single sequence, number of times below zero, and lowest recorded temperature.

Site	Model Location	Number of temperatures below 0°C	Mean (±SD) (hours)	Minimum and maximum (hours)[1]	Minimum and maximum temperatures (°C)
Calafate	Exposed	177	21.5 (102.5)	1-1266	-8.91 to 44.89
	Under a tussock (*Mulinum spinosum*), near the roots	7	463.5 (1050.5)	9-2937	-1.97 to 31.93
	Buried ~10 cm	13	276 (928)	2-3427	-3.37 to 32.76
	Buried ~15 cm	194	3 (18.5)	1-257	-1.97 to 30.71
Esquel	Exposed	118	7.5 (7)	1-43	-8.70 to 48.37
	Under a tussock (*Mulinum spinosum*), near the roots	69	8 (8.5)	1-45	-6.58 to 39.32
	Buried under a bush ~10 cm	25	13 (15)	1-72	-4.56 to 30.70
	Buried under a rock of ~40 cm diameter	23	9 (7)	1-21	-3.33 to 30.69
	Buried ~10 cm	7	20.5 (34)	2-100	-1.61 to 30.90
	Buried ~15 cm	7	26 (33)	8-103	-2.88 to 30.67

[1]**Minimum and maximum number of consecutive hours with temperatures below 0°C in a single sequence.**

In heating refuge degree-day for 0°C, the refuge with fewer degree-days below 0°C in Calafate was buried ~ 15 cm in the ground (9.15 degree-days); and the refuge with the most degree-days below 0°C was buried ~ 10 cm in the ground (108.78 degree-days). Meanwhile, in Esquel, the refuge with fewer degree-days below 0°C was buried ~ 10 cm in the ground (3.34 degree-days); and the refuge with the most degree-days below 0°C was placed under a bush (21.95 degree-days; χ^2_{7} test= 1611,1; p< 0,001; Figure 1).

Supercooling point (SCP) and Critical Thermal Minimum (CT_{Min})

Lizards from Calafate showed freezing exotherms at a mean temperature of -7.54 ± 0.49°C, while the supercooling point for lizards from Esquel was higher, at -5.80 ± 0.82°C (Table SI; ANOVA: $F_{1; 9}$=17.155; p=0.002). No lizard survived the slow thaw after experiencing the exothermic freezing reaction, neither from Calafate nor from Esquel.

Lizards showed a CT_{Min} ranging from 2.23 to 5.86°C for Calafate's individuals and from 3.97 to 6.21°C for individuals from Esquel. Calafate individuals (mean= 4.30 ± 1.29) had lower CT_{Min} than Esquel individuals (mean= 5.10 ± 0.61; ANOVA: $F_{1; 23}$= 4.500; p= 0.004).

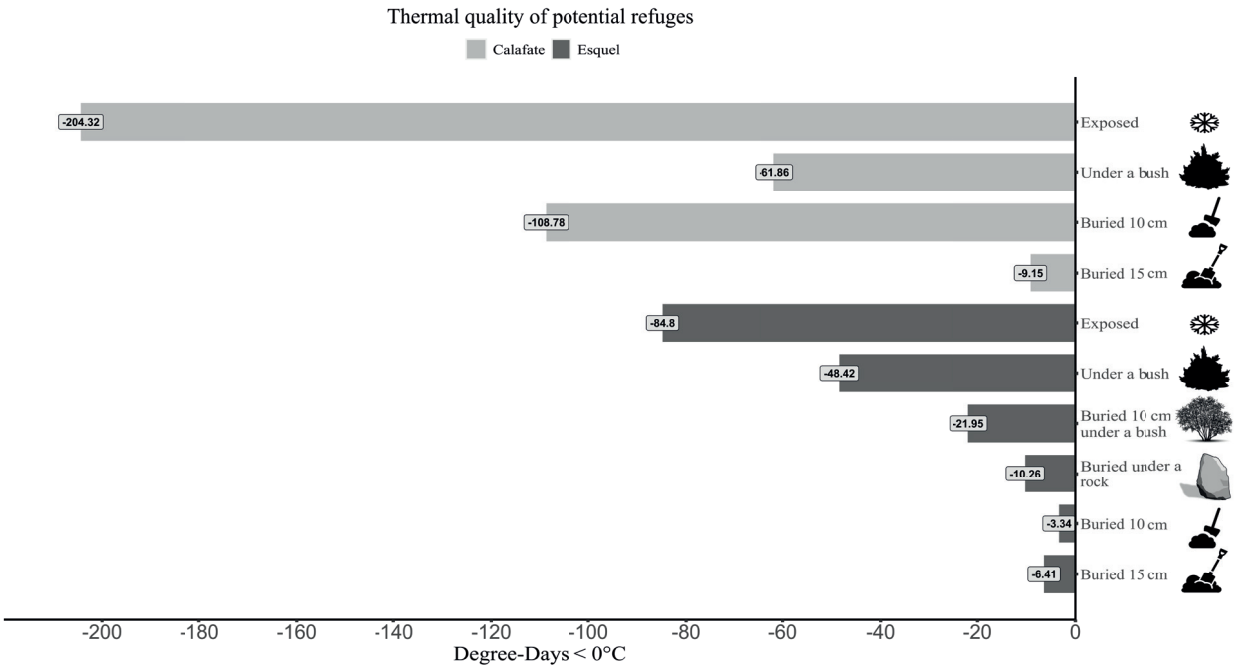

Figure 1. Thermal quality of the potential winter refuges (degree-days) in Calafate (light grey) and Esquel (dark grey). Values for degree-days below 0°C are represented for each potential refuge.

Control and cooled-down individuals, before and after the cooling experiments

During the cooling experiment, we did not detect an exothermic reaction from any individual from Calafate or Esquel and, after removing lizards from the plastic containers, we found no ice or evident sign of freezing (such as rigidity of the animals or a change in the colour of their skin). Additionally, individuals reacted seconds after we took them out of the freezer (except for lizards from the first Esquel experiment, that were found dead), although in a seemingly lethargic state, with slow movements.

The control individuals from Calafate showed negative values of adjusted glucose change (ΔAGluc: mean= -22.7 %) and individuals that were cooled down showed positive values (mean= 8.9%; ANOVA: $F_{1; 18}$= 126.24; p < 0.001). For adjusted lactate change, no differences were found between control (mean= -33.35 %) and cooled-down individuals (mean= 0.29 %; ΔALac: ANOVA, $F_{1; 18}$=1.79; p=0.20). A comparison of ΔAGluc between control groups of both populations showed no significant differences (ANOVA, $F_{1; 17}$= 0.31; p= 0.59).

Control individuals from the Esquel experiment showed negative values of ΔAGluc (mean= -19.49 %) and individuals that were cooled down showed positive values (mean= 26.68%; χ^2_{24} test= 54.39; p< 0.001). In the "milder complementary experiment", control individuals showed negative values of ΔAGluc as well (mean= -36.64 %; N= 3) and cooled-down individuals positive values (mean= 32.96%; N= 6). For ΔALac, control individuals showed significantly lower increases (mean= 12.16 %) than cooled-down individuals (mean= 387.28 %; χ^2_{24} test= 31.99; p< 0.001). Meanwhile, in the "milder complementary experiment", control individuals showed negative values of ΔALac (mean= -37.54 %, N= 3) and cooled-down individuals an increase (mean= 201.83%, N= 6; Figure 2, Table SII).

The urea, total proteins, and albumin, which were measured only after the experiments from Calafate, did not show significant differences

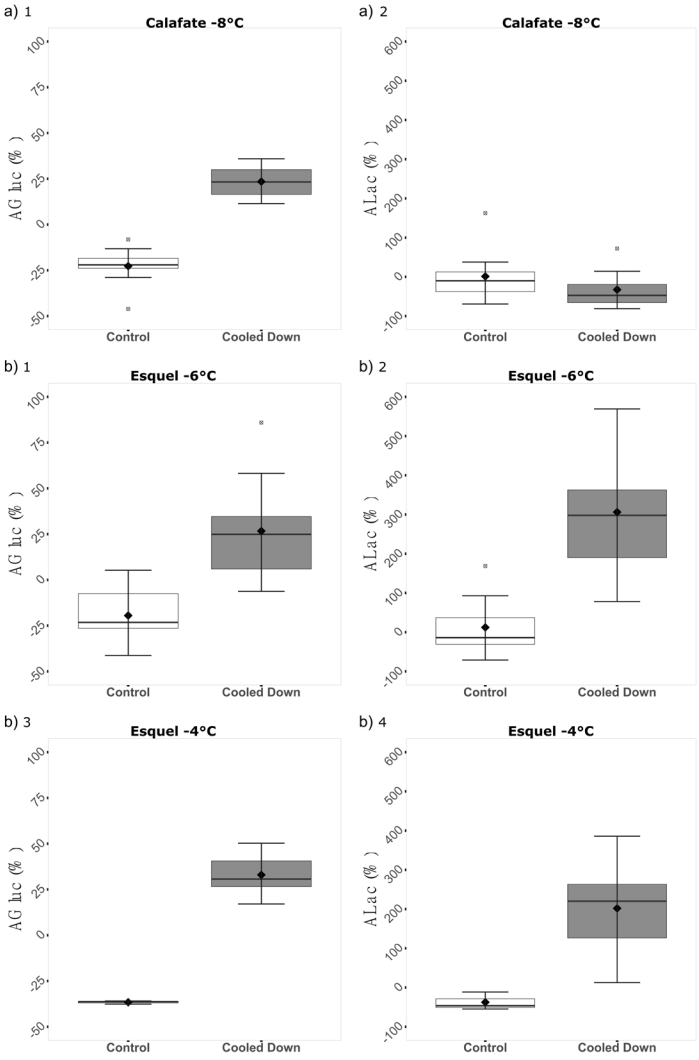

Figure 2. Results from the Adjusted Glucose (ΔAGluc) and Adjusted Lactate (ΔALac) analyses corresponding to the Calafate and Esquel cooling experiments. Values for a) individuals from Calafate ($N_{cooled\ down}$= 10; $N_{control}$= 10) for ΔAGluc and ΔALac; and b) individuals from Esquel ($N_{cooled\ down}$= 10; $N_{control}$= 9), for ΔAGluc and ΔALac from the first experiment (1-2) and the milder complementary experiment (3-4; $N_{cooled\ down}$= 6; $N_{control}$= 3). Median (black horizontal line) and mean (rhombs) are represented in all groups. The middle 50% of values are inside each box; whiskers represent upper and lower quartiles.

between controls and cooled-down individuals (Table III).

DISCUSSION

Where to spend the winter seems to be crucial in how *Liolaemus* lizards are coping with the dangers of low temperatures in Patagonia. Populations of *L. lineomaculatus* from this study were not able to tolerate freezing but survived cold exposure with low supercooling points comparing with other reptile species of similar environments (-7.5°C for lizards from the cold site, Calafate, and -5.8°C for lizards from the temperate site, Esquel). Results from the biochemical analyses showed increases in concentration only in glucose after cold exposure, in possible association with cold hardiness mechanisms. However, the increase in concentration is probably not enough to elevate osmolality in an ecologically significant way, considering similar experiments (Costanzo et al. 1991) with external glucose loading, where survival was increased when concentrations in plasma reached over 50 μmol*ml^{-1} (in our experiments, values ranged between ~5 and ~15 μmol*ml^{-1}). Lizards could spend a very short time at temperatures near or below their population's SCP in any of the potential refuges analysed in this study. Furthermore, there was a correlation

Table III. Comparison of biochemical variables between control (n = 10 for both Calafate and Esquel) and cooled down individuals (n = 10 for Calafate and n = 9 for Esquel) of *Liolaemus lineomaculatus*. In the case of urea, values include the significant covariable SMI ($F_{1; 10}$ = 10.356; p = 0.001). All means are expressed in g/L. Analyses were performed as ANCOVAS.

Site	Variables	*F*	*p*	Control means (±*SD*)	Cooled down means (±*SD*)
Calafate	Urea (heart) n = 20	0.065	0.802	0.11 (0.07)	0.12 (0.11)
	Urea (liver) n = 20	0.031	0.862	0.12 (0.08)	0.12 (0.08)
	Total proteins (heart) n = 20	0.225	0.641	18.50 (8.69)	20.28 (8.10)
	Total proteins (liver) n = 20	3.526	0.077	132.05 (54.15)	172.81 (42.18)
	Albumin (heart) n = 20	0.564	0.463	10.94 (1.84)	9.96 (3.69)
	Albumin (liver) n = 20	1.959	0.181	45.5 (6.09)	49.56 (10.64)
	Initial glucose n = 20	-	-	1.97 (0.22)	1.88 (0.22)
	Final glucose n = 20	-	-	1.52 (0.29)	2.31 (0.26)
	Initial lactate n = 20	-	-	0.41 (0.28)	0.32 (0.18)
	Final lactate n = 20	-	-	0.31 (0.16)	0.16 (0.07)
Esquel	Initial glucose n = 19	-	-	1.85 (0.30)	1.71 (0.44)
	Final glucose n = 19	-	-	1.46 (0.14)	2.09 (0.38)*
	Initial lactate n = 19	-	-	0.27 (0.08)	0.48 (0.19)
	Final lactate n = 19	-	-	0.29 (0.19)	1.70 (0.23)*

All means are expressed in g/L. Analyses were performed as ANCOVAS with SMI as a covariable, if significant. Initial and final glucose were not analyzed because the change in glucose was analyzed as ΔAGluc (%), and the same was the case for lactate, analyzed as ΔALac (%).
***These values were obtained from lizards found dead after the experiment.**

between cold hardiness and severity of weather or thermal quality of potential refuges, as we expected. Lizards from Calafate, where the PVC models were exposed to cold temperatures or more frequent cold spells showed a lower mean CT_{Min} and were able to supercool with a lower SCP than lizards from Esquel. Furthermore, lizards from Esquel did not survive exposure to temperatures near their SCP, even when they did survive the exposure to temperatures below 0°C for several hours. It is very likely that *L. lineomaculatus* relies mostly on use of appropriate refuges rather than physiological mechanisms to overwinter in Patagonia, as does *L. pictus* (Cecchetto et al. 2019), although probably with a higher capacity to endure a cold climate, reaching a lower SCP (-5°C; Cecchetto 2021) and lower CT_{Min} (6.9°C, Kubisch et al. 2011).

The actual thermal regime experienced by ectotherms may be more heterogeneous than predicted by only latitude or elevation (Ficetola et al. 2018), but results from the potential-refuges showed that lizards living in Esquel are more likely than those in Calafate to find and use microhabitats that are better thermally buffered (*e.g.,* under rocks or within rock crevices). There was variation between populations in critical thermal minimum (CT_{Min}): individuals from Calafate had a lower CT_{Min} than individuals from Esquel. This is consistent with several studies showing CT_{Min} for ectotherms, varying across environments with different cold

regimes (Hoffmann et al. 2002, Huang & Tu 2008, Moritz et al. 2012, Clusella-Trullas & Chown 2014), suggesting that CT_{Min} is physiologically relevant and could directly affect survival at cold and temperate habitats (but see also Winne & Keck 2005, Du 2006, Yang et al. 2008).

The CT_{Min} values of both populations of *L. lineomaculatus* were among the lowest values recorded for liolaemids from Patagonia (Bonino et al. 2015, Kubisch et al. 2016), which is not surprising given that this species is one of the southernmost lizard species in Argentina.

The values of supercooling points (SCP) obtained from these *Liolaemus lineomaculatus* populations fall in the range of other lizards such as *Uta stansburiana* (varying among populations, between -7°C and -10°C , Michels-Boyce & Zani 2015), *Eulamprus tympanum* and *E. kosciuskoi* (-6.5°C and -8.5°C, respectively; Spellerberg 1972), and *Podarcis muralis* (-5°C; Claussen et al. 1990). Furthermore, we also found variation between populations in supercooling points: lizards from the cold site, Calafate, showed a mean value lower than lizards from the temperate site, Esquel. Notably, only the Calafate population was able to survive for several (12) hours at near SCP temperature. Variable cold hardiness has also been reported for *Zootoca vivipara,* which not only showed different temperatures of crystallization in populations from different habitats (Voituron et al. 2004, Berman et al. 2016), but also the possibility to alternate between freeze tolerance and supercooling (Grenot et al. 2000, Voituron et al. 2002). Interestingly, in the case of *Uta stansburiana,* among the 12 populations sampled, there was no correlation between winter harshness and supercooling points (Michels-Boyce & Zani 2015). Future endeavours could focus on sampling more populations of *L. lineomaculatus* to determine if the association between thermal quality of the sampling sites and cold hardiness found in the present study persists as a trend related to environmental restraints.

Consistent with results obtained for *L. pictus* (Cecchetto et al. 2019), we did not find any significant differences between control and cooled-down individuals for urea, total proteins, or albumin. Urea has been associated with cold hardiness by increasing the plasma osmolality of some reptiles such as hatchlings of *Chrysemys picta* (Costanzo et al. 2000) and some amphibians such as *Lithobates sylvaticus* (Costanzo & Lee 2005), but the evidence suggests that it is not directly involved in the cold hardiness mechanisms of *L. lineomaculatus*. On the other hand, the search for antifreeze proteins (AFPs) in the blood of ectotherms, other than fish, has not yet yielded positive results. Researchers have tried to find AFPs in freeze-tolerant wood frogs (*Lithobates sylvaticus;* Wolanczyk et al. 1990), turtle hatchlings (*Chrysemys picta;* Storey et al. 1991 and *Chelydra serpentine;* Costanzo et al. 2000), and the European common lizard, *Zootoca vivipara* (Voituron et al. 2002), without success. Given the scarce information regarding AFPs in liolaemids, the search for potential proteins related to cold hardiness is worthy of research. Nevertheless, we found no evidence of AFPs as part of the mechanisms used in *L. pictus* (Cecchetto et al. 2019) or *L. lineomaculatus* to survive winter in Patagonia.

Cooled-down individuals of *L. lineomaculatus* from both Calafate and Esquel showed an increase in blood glucose during experiments, while control individuals showed a general decrease. Lactate, on the other hand, did not present such a clear pattern. Final lactate concentration in individuals from Calafate was also almost 10 times less than that in individuals from Esquel, which could be due to mechanisms regulating the acid-base homeostasis that could not begin in the Esquel population since those

individuals died from the cold. Additional work with larger sampling would be necessary to fully understand the cold hardiness in this species. However, in individuals from Esquel, there was an increase in lactate for cooled-down individuals and a decrease in controls. While it is tempting to associate the glucose increase in cooled-down individuals with a cold hardiness response, the small concentration of this increase suggests that glucose for this species may not be specifically associated with colligative cryoprotection, in the same way as Voituron et al. (2002) concluded for *Zootoca vivipara* (where concentrations reached ~25 $\mu mol*ml^{-1}$, while values obtained in our experiments ranged between ~5 and ~15 $\mu mol*ml^{-1}$). Furthermore, the increase in lactate in cooled-down individuals from Esquel could be indicating that the contribution of glucose in elevating osmolality may be secondary to its role in anaerobic energy metabolism. The role of glucose as a metabolic fuel in anaerobic metabolism during periods where low temperature slows or halts oxygen circulation is well known (Calderon et al. 2009, Sinclair et al. 2013), especially for organs like the brain, which relies on glucose derived from the liver glycogenolysis during anoxia (Clark & Miller 1973). Thus, we consider that the role of glucose in *L. lineomaculatus* at cold temperatures is mainly related to maintaining metabolism despite cold-induced anoxia and perhaps protecting cells by limiting cell dehydration.

Vegetation structure and land topography can cause big differences in soil temperature and snow disappearance over short distances (Ford et al. 2013). Here, we explored the thermal quality of potential refuges for lizards in small areas of each sampling site, representing a sample of the options individuals might choose every year when winter comes. Previous analyses from potential refuges for *L. pictus* showed alternatives where lizards could spend most, if not all winter above 0°C (Cecchetto et al. 2019). *Liolaemus lineomaculatus* inhabits colder environments than *L. pictus* (at higher elevations or latitudes); it is, therefore, unlikely that it could spend most winter above 0°C. Even though the thermal quality of potential refuges varied greatly at each site, and between Calafate and Esquel, potential refuges rarely remained at temperatures near or below each population's SCPs. We found that, despite being buried at ~10 -15 cm, lizard models were well buffered from air temperatures at the selected potential refuges, which is consistent with previous works that found that 10 cm of soil caused significant thermal buffering where below-ground raiding species were collected (Baudier et al. 2015). It should be pointed out that, while the homogeneity of the environment allowed us to cover the most representative microenvironments with few PVC models, the relatively low number of models used in this study did not allow for replicate measurements of each potential refuge at each site. Appropriate refuge selection is most likely what allows individuals of *L. lineomaculatus* to survive the winters without heavily investing resources in costly physiological mechanisms, preserving those resources for the sporadic heavy winter spells. In this viviparous species, saving energy can be vital, considering that females give birth to 3-4 individuals between late summer and the beginning of autumn and post-partum females start brumation in early autumn (Medina & Ibargüengoytía 2010).

Liolaemus lineomaculatus occupies locations with harsher cold climates than *L. pictus* in the highlands and high latitudes of Patagonia and, unlike *L. pictus*, this species seems to be able to supercool. This ability to supercool appears to be related to the cold regime of the location, varying between populations, although further studies are needed to determine if it is a result of adaptation or plasticity. In our study, we could

not find evidence of biochemical metabolites that explain the endurance of *L. lineomaculatus* to live in one of the coldest environments for Liolaemidae, except for a small increase in glucose. While potential refuges analysis for *L. lineomaculatus* revealed that suitable refuge selection must be key in the survival of lizards in the winters of Patagonia, perhaps even more so than any physiological mechanism. However, the threat of reduced snow deposition caused by global warming might force lizards to rely on plastic physiological and behavioural responses to survive the winter at the risk of depleting energy reserves. This work provides information and results on the physiological and ecological aspects of the question: "How is a 15-cm lizard able to endure the cold in the highlands and high latitudes of Patagonia, Argentina?" However, further work to discover what is going on under the snow with ectotherms in temperate and cold environments is needed.

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SUPPLEMENTARY MATERIAL

Tables SI, SII, SIII
Figure S1

How to cite

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