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Freeze tolerance in neotropical frogs: an intrageneric comparison using *Pristimantis* species of high elevation and medium elevation

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Research Article

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Abstract

Paramos are high-elevation tropical Andean ecosystems above the tree line that display variable temperature and frequent freezing spells. Because a significant anuran community lives in this environment, physiological protection against freezing must characterise individuals in this community. Antifreeze protection has been studied in amphibians from other communities, and it is likely that Paramo anurans rely on the same underlying molecules that convey such protection to Nearctic species. However, given the pervasive presence of freezing spells in the Paramos year-round, the processes of activating protection mechanisms may differ from that of seasonal counterparts. Accordingly, this study investigated cryoprotection strategies in high-elevation tropical frogs, using as a model the terrestrial and nocturnal genus *Pristimantis*, specifically *P. bogotensis*, *P. elegans* and *P. nervicus* from Paramos, and the warm ecosystem counterparts *P. insignitus*, *P. megalops* and *P. sanctaemartae*. We focused on freeze tolerance and its relationship with glucose accumulation and ice formation. Under field conditions, the highest elevation *P. nervicus* exhibited higher glucose concentration at dawn compared to noon (1.7 ± 0.6 mmol/L versus 3.5 ± 1.32 mmol/L). Under experimental thermal freeze exposure for 2 hours between −2 and −4 °C, the glucose concentration of the three Paramo species increased but physiological diversity was evident (*P. nervicus* 126%; *P. bogotensis* 100%; and *P. elegans* 55%). During this test, body ice formation was assessed calorimetrically. The species with the highest body ice formation was *P. bogotensis* (17% ± 5.37; maximum value: 63%; n = 8), followed by *P. nervicus* (5% ± 3.27; maximum value: 11%; n = 5) and *P. elegans* (0.34% ± 0.09; maximum value: 1%; n = 4). The study shows physiological diversity both within a genus and across the amphibian community around the freezing contour. Overall, Paramo species differ in freezing physiology from their low-elevation counterparts. Thus, climate shifts increasing freezing spells may affect the structure of communities in this zone.

Introduction

Environments reaching subzero temperatures limit faunal diversity in species able to avoid or tolerate freezing. Whereas this phenomenon may be deleterious to various extents as determined by body size and physiology, the ability to survive partial freezing has evolved independently in various vertebrate lineages (Marchand 1996; Storey and Storey 2017, 1996). Most reported cases scrutinise animal species at high latitudes and altitudes that display seasonal freezing (Cabrera 1996; Sandercock et al. 2005); yet, the ecology of freezing exposure is highly diverse (Davenport 1992; Costanzo and Lee 2013). Depending on the environment, exposure to freezing spells may differ in spatial and temporal thermal variance, predictability, seasonality, duration and absolute temperatures. For example, the Nearctic climate involves seasonal exposure to freezing, is typically associated with regulated and periodic inactivity and concomitant metabolic depression, as occur since hundreds of species of terrestrial insects (especially among Hymenoptera, Diptera, Coleoptera and Lepidoptera), until famous freeze tolerance vertebrates as the wood frog *Lithobates sylvaticus* (formerly *Rana sylvatica*) or the garter snake *Thamnophis sirtalis*, among others (Storey and Storey 1996). In contrast, tropical high elevations such as the Paramos (tropical Andean ecosystems above the tree line) encompass episodic freezing conditions lasting from a few minutes to hours (Carvajalino-Fernández et al. 2011), a pattern that is sustained all year round, from a few nights to every night, according to local nuances. In addition, such environments display enhanced temporal variance in temperature and extensive diel thermal ranges that may involve freezing spells and warm temperatures over short periods of

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time (Navas et al. 2013). Due to the phenomenon termed "night sky radiation cooling" (Dobson 2005), such spells are more frequent on unclouded nights, when several species are most active. Tropical high-elevation amphibian communities have not been studied from the standpoint of freezing biology, but a number of species of Nearctic anurans have been investigated regarding patterns and subjacent processes (Layne and Lee 1995). The best-studied species is probably *L. sylvaticus,* but data are also available on *Hyla chrysoscelis, H. versicolor, Pseudacris crucifer* and *P. triseriata* (Schmid 1982; Storey and Storey 2017, 1992; Layne and Jones 2001; Costanzo and Lee 2013).

One antifreeze mechanism common to anuran species studied so far is the accumulation of carbohydrates with low molecular weight, such as glucose, which enhances cryoprotection, perhaps as a reactive response to internal ice formation (Voituron et al. 2009a). Such carbohydrates, either alone or interacting with other molecules, elevate the osmolarity of body fluids, thus decreasing cell shrinkage during extracellular ice formation (Storey and Storey 2017). In addition, elevated levels of glucose augment tolerance to ischaemia by maintaining ATP turnover under hypoxic conditions and favour metabolic arrest (Storey and Storey 1996; Voituron et al. 2009b). The frequency of freezing and thawing events can also enhance cryoprotectant production, given that rapid sequences of cooling and warming rates promote survival in *L. sylvaticus* exposed to freezing (Larson and Barnes 2016). In parallel, Ice-Nucleating Proteins (INPs) control the formation of ice in extracellular fluids (Storey and Storey 1992a), so that the scope of body ice formation relates to both the density of cryoprotectants and the duration of the exposure to freezing temperature (Clausen and Costanzo 1990). It is probable that, in terms of active molecules, the mechanisms just described characterise all freeze-tolerant amphibians, including those in the Paramos. Yet, the nature of responses (e.g., reactive, cyclic or permanent) and the basal line of cryoprotection may vary with the ecology of freezing (Rexer-Huber et al. 2015), particularly given the daily exposure to freezing in the Paramo species.

The cryoprotective process of temperate anurans involves differential gene expression in pre-wintering frogs as a preparation to freezing conditions to come (Kiss et al. 2011; Storey and Storey 2017). However, such seasonal patterns of expression would be at best secondary for species exposed to freezing on a daily basis, such as Paramo amphibians. Because this habitat is home to at least 40 species representing several genera and families (Bernal and Lynch 2008), it seems reasonable to infer that all of them experience, at least occasionally, nocturnal freezing spells followed by higher temperatures during the day (Carvajalino-Fernández et al. 2011). Accordingly, we postulate that Paramo anurans display ecological, behavioural and physiological traits compatible with freezing. However, previous observations suggest that avoidance mechanisms may not typify Paramo species, at least not as a valid generalisation. For example, frogs in the genus *Pristimantis* (which are well represented in Paramos) tend to be nocturnal and terrestrial and call from exposed sites, thus experiencing the coldest possible conditions during activity time (Navas 1996a). Although Paramos offer thermal diversity across the landscape and some microhabitats offer some thermal protection, avoiding frozen risk on the coldest nights may not be possible (Navas 1996a; Carvajalino-Fernández et al. 2011). Inactive individuals may be exposed to very low temperatures, and even fully active individuals have been reported at temperatures as low as −5 °C (Navas 1996b; Lüddecke and Sanchez 2002; Carvajalino-Fernández et al. 2011). Consequently, activity temperatures in some Paramo species may match those that simulate hibernation in *L. sylvaticus* (Costanzo et al. 1991).

This study is a first attempt to explore physiological freeze tolerance in high-elevation tropical anurans, using as a model Paramo species in the genus *Pristimantis*. These Paramo frogs experience freezing conditions resembling those suffered by some Nearctic amphibians at the beginning of spring, when they engage in breeding, despite the freezing risk (Storey and Storey 1987). Because *Pristimantis* frogs from Paramos live permanently under such conditions, our hypothesis is that these high mountain amphibians, when exposed to risk events of freezing, present an increase in glucose (as a cryoprotectant) and internal freezing of fluids, characteristics associated with freeze tolerance strategy (Schmid 1982; Storey and Storey 1996; 2017). Additionally, because this genus originated before the major uplift in the tropical Andes (Heinicke et al. 2007), freezing tolerance is unlikely to be an ancestral trait. Therefore, we also expected freeze tolerance to be an exclusive (or more evident) characteristic of *Pristimantis* montane species that live above the freezing line. To test our hypotheses, we conducted a study focusing on the species *P. bogotensis*, *P. elegans* and *P. nervicus*, with partial comparison with lower elevation counterparts. This comparison is not only relevant to the enhancement of knowledge of freeze tolerance in animals but may also contribute to explaining climate-related changes in the structure of tropical amphibian communities near the tree line (Navas 2003; Navas et al. 2013).

Materials and methods

Localities and animal care

We conducted part of this study at the Buitrago area of the Chingaza National Park, located in the highlands of the eastern branch of the Colombian Andean Cordilleras. This Park ranges between 3400 m and 3700 m and has its central point located at 4° 44′ 38″ N and 73° 50′ 11″ W. The Paramo is the ecosystem present in this locality, characterised by a cold and humid climate, with sudden changes in the atmospheric state, and although the annual temperature fluctuation is small (2 to 10 °C), the thermal amplitude during the day varies from below the freezing point (environmental night temperatures below −15 °C) to 40 °C (Sarmiento 1986; Madriñan 2004; Carvajalino-Fernández et al. 2011). Detailed information on daily temperature shifts in different microhabitats at the locality of work has been published elsewhere (Navas 1997; Navas et al. 2013; Carvajalino-Fernández et al. 2011). It is typical that the coldest night temperatures and the lowest precipitation levels occur simultaneously (between 9 and 17% of annual rainfall, see Vargas and Pedraza 2004).

The other location of the study was the upper basin of the "Gaira" River, on the north-western slope of "Sierra Nevada de Santa Marta", an area known as "La Cascada" in the province of "Magdalena" in Colombia. This area is located at 1560m and has its central point placed at 11° 10" N, 74° 10′ 45". The locality is characterised by a tropical montane cloud forest with average annual rainfall of 2446 mm and steep slopes of primary and secondary forest in advanced state of regeneration, with temperatures from 14 to 24 °C (Hernández-Camacho and Sánchez-Páez 1992; Rueda-Solano et al. 2016). There is a high diversity of microhabitats in the sector, including rocky areas, sand and leaf litter on the banks of the stream. Inside the riparian forest, undergrowth is found within about 10 m distance from the stream basin. The soil in the forest is covered mostly by leaf litter and dead wood, and

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there are fern patches where the tree cover is sparse (Carvajalino-Fernández et al 2012).

Regarding animal collection for data gathering, we divided the samples into two groups: one for field and the other for laboratory experiments:

Animals used in field work

From the Paramo locality, we obtained samples from *P. bogotensis* (n = 4; weight = 1.2 ± 0.22 g), *P. elegans* (n = 10; weight = 4.1 ± 1.83 g) and *P. nervicus* (n = 7; weight = 1.15 ± 0.25 g). From the tropical montane cloud forest locality, we collected the frogs species *P. insignitus* (n = 11; weight = 2.64 ± 0.91 g), *P. megalops* (n = 12; weight = 1.9 ± 0.73 g) and *P. sanctaemartae* (n = 10; weight = 3.62 ± 1.15 g). For *P. insignitus*, *P. megalops* and *P. sanctaemartae*, all the specimens found were juveniles, whereas all other cases were adult males. The "n" values above do not indicate the total number of animals that could be collected, but the number of individuals from which adequate blood samples could be collected. Unfortunately, we were constrained by low effective "n" values because of the extreme difficulty involved in obtaining valid samples for certain species, and the previously established protocol preventing re-use of animals that could not be sampled in a first attempt, all this in the context of permits limiting the total number of animals that could be collected. As a result, the final N values are more related to data collection issues, not to abundance in the field. For example, despite being a common species, field samples derived from cardiac puncture were particularly challenging in *P. bogotensis* given their small size, and the rapid clotting (and even freezing) of blood samples. We collected the paramo species from December 2009 to January 2010 and tropical montane cloud forest in October 2009.

Animals used in laboratory work

For laboratory experiments, we collected just animals from the Paramo locality; these were *P. bogotensis* (n = 36; weight = 1.11 ±0.32 g), *P. elegans* (n = 20; weight = 5.33 ± 1.9 g) and *P. nervicus* (n = 25; weight = 1.4 ± 0.25 g). We maintained specimens in a laboratory in Bogotá, at 2600 m, where terrarium temperatures ranged from 8 to 17 °C, mirroring trends outdoors. The light cycle was artificially set to 12:12 h daytime/nighttime, and frogs had access to water and arthropods for food ad libitum. All frogs appeared normally active during captivity. The experiments were performed up to three days after capture, and frogs were not used in experiments after 10 days in the laboratory.

Controls

The experiments reported here lead to unambiguous interpretations only if adequate controls are performed. Ideally one should control for a "first test effect" by randomising order when comparing day and night patterns or alternative time-of-day related periods. In addition, controls should be performed to isolate the effects of puncturing, manipulation, time in captivity and several other variables. Whereas we are familiar with such formal aspects of experimental science, our study animals were wild species collected in small numbers (details in *Localities and animal care)*. Accordingly, we prioritised a control for manipulation effects applied only to laboratory tests (described below under *Glucose concentration change by freeze exposure*). This was considered the most general and informative procedure, and it was applied to five individuals of *P. bogotensis*. To these specimens, we applied a protocol that preserved all the manipulation and dual puncturing of the freezing protocol but did not subject animals to cold exposure (freezer off, same time in). It turned out that no differences in the glucose level could be detected in animals before nor after this manipulation-only protocol (Wilcoxon paired test: Z: 2.02; p: 0.053; maximum value of glucose: 1.83 mmol/L). Other species may display similar patterns, but we acknowledge this as a supposition to be confirmed. We also controlled for experimental order (randomised) when such procedures did not require an increase in the number of animals.

Body temperature and glucose concentration: night-day contrast

To verify if frogs in the field display enhanced glucose concentration at night when temperatures are lower, we compared paired samples of glucose concentration obtained from blood extracted by cardiac puncture. We applied this protocol to the same individual at 12:00 and at 04:00 hours. We randomised the extraction order, which was applied first at 12:00 hours in some individuals, and at 04:00 hours in others, randomly. Frogs were fasted in a field terrarium during 24 hours prior to sampling, a protocol designed to reduce variance in glucose concentration induced by feeding history. The time of day selected for sampling was that maximising thermal differences according to our field temperature records. Before starting glucose sampling, body temperatures were measured with an infrared thermometer IR201 Extech 6:1. Field blood samples (0.7 µl) were collected by cardiac puncture, and the glucose level was measured with a glucometer (Alphatrak, Abbott Laboratories). The glucometer function was calibrated every five measures with the AlphaTRAK Control Solution (Alphatrak, Abbott Laboratories).

The influence of body mass on glucose modulation between treatments was evaluated, for each species, using ANCOVA tests, performing them with the packages tidyverse (Wickham et al. 2019), ggplot2 (Wickham 2016), ggpubr (Kassambara 2020), rstatix (Kassambara 2021) and broom (Robinson et al. 2021) of the statistical environment R (version 3.6). Body temperature and glucose data were compared intraspecific using Wilcoxon paired test. In addition, we explore differences interspecific of the body temperatures and glucose concentration, recorded during dawn, using a Kruskal–Wallis tests and multiple comparisons with Bonferroni correction tests. The analyses above mentioned were performed by applying the package ggplot2 (Wickham 2016) of the statistical environment R (version 3.6).

Glucose concentration change by freeze exposure

To analyse physiological responses to freezing, we followed blood extraction and glucose analysis protocols similar to those reported in 2.3, although in the context of experiments beginning at 20:00 (night period). From each specimen, we 1) took a glucose sample, 2) moved it to a refrigerator (5 to 8 °C) for one hour (to simulate a Paramo temperature drop), 3) transferred it to an airtight 50 ml top jar and, in turn, submerged it in a salted ice bath at −3 °C for two hours. At this final stage, the expected drop in body temperature was recorded using the NTC thermistor referred to in section (2.5). After procedure 3, we obtained a second glucose sample. The time lapse between sample collections was about three hours. After the second sample, frogs were moved to individual containers kept at 12 – 17 °C for two days. Time recovery was set when the frogs regained the regular breathing, normal posture and righting response, after removing from the container used for freezing treatment. Only after two days were frogs considered recovered

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from the treatment; we compare species recovery time using ANOVA test and Tukey post hoc test of the statistical environment R (version 3.6). The influence of body mass on the changes of glucose levels was explorer using the same methodology reference in point 2.3 of this manuscript. We performed Wilcoxon pair tests for group comparison using the statistical environment R (version 3.6) as in the others data analysis.

Ice quantification protocol

We quantified body ice formation inside the frog after the freezing protocol described above. We used a glass covered container (10 ml) fitted with glass capillary tubing in the middle of a calorimetric cell whose calorimetric constant could be determined by Joule heat calibration using a 10Ω resistor. All the calorimetric system was insulated with rigid foam (polystyrene). We calculated the exothermic heat produced, during the freezing experiment in the frogs, according to the enthalpy of melting ice by generating a temperature curve with a previously calibrated NTC thermistor of 10 KΩ. This thermistor was connected to a Multimeter Multimaster 570 True RMS (Extech Inc.) able to register data each second with the BS85X software programme (Version 5.1.0.4). Calculations required information on the water content of each species, so we euthanised five frogs of each species with an overdose of 2% lidocaine gel and dried them to constant weight at 70 °C (Schmid 1982). Body water content was expressed as a percentage of wet mass and as the mass of water per dry body mass (g H2O/g dry wet; adapted from Layne & Jones 2001). The quantity of frozen water was calculated in individual specimens from the enthalpy of the melt curve (Schmid 1982) using the Origin7 programme (Originlab, version 7).

Results

Body temperatures under restrained field conditions

In terraria placed in the field and exposed to natural variation in temperature, body temperature of all the species decreased at night (Wilcoxon paired test: *P. insignitus*, W: 110, p<0.01; *P. megalops*, W: 168, p<0.01; *P. sanctaemartae*: W: 132, p<0.01; *P. bogotensis,* W: 47, p<0.01; *P. elegans,* W: 210, p<0.01; *P. nervicus* W: 42, p<0.01; Figure 1). Besides, body temperatures of paramo frogs were substantially lower at dawn (04:00 am) that those of their montane rain-forest counterparts at analogous conditions (KW: 47.83; p<0.01; Figure 1).

Glucose modulation in field and in laboratory

Outcomes found in the field

Body mass did not influence the glucose modulation in experiments performed in the field with both Paramo and tropical montane cloud forest species (ANCOVA: *P. insignitus,* F: 4.12, p: 0.06; *P. megalops,* F: 1.06, p: 0.32; *P. sanctaemartae*, F: 1.33, p: 0.28; *P. bogotensis,* F: 1.48, p: 0.31; *P. elegans,* F: 0.31, p: 0.58; *P. nervicus*, F: 0.149, p: 0.713). Glucose levels of Paramo frogs were substantially higher at dawn (04:00 am) that those of their tropical montane cloud forest counterparts at analogous conditions (KW: 47.83; p<0.01; Figure 2). In terms of diel cycles, *P. bogotensis* and *P. nervicus* displayed a substantial increment in glucose levels when compared measures taken at noon and during the dawn (Wilcoxon paired test: *P. bogotensis,* W: 21, p< 0.03, an increase of 64%; *P. nervicus,* W: 27; p: 0.02; an increase of 104%; Figure 2).

Outcomes found in the laboratory

There was also no association between body mass and glucose modulation in the data registered in Paramo species in the laboratory (ANCOVA test: *P. bogotensis,* F: 1.64, p: 0.25; *P. elegans,* F: 2.04, p: 0.17; *P. nervicus*, F: 1.24, p: 0.302). Freezing induced in the laboratory caused an increase in blood glucose concentration in all paramo species (Wilcoxon paired test for all the species: *P. bogotensis,* W: 121, p< 0.01, an increase of 100%; *P. elegans,* W: 127.5, p< 0.01, increase of 55%; *P. nervicus*, W: 104, p: 0<0.01, increase of 126%; Figure 3).

Recovery patterns after experimental freezing in Paramo frogs

All Paramo frogs recovered from freezing, in a maximum of two days after experimental freezing, with locomotion and feeding patterns seemingly comparable to those of untreated animals. However, for quantification purposes, we considered "time to recover" as the time lapse between the end of the experiment (i.e., the time when frogs were removed from the calorimeter) and the time at which animals displayed regular breathing, normal righting response and ability to jump after light prodding. Species showed differences between times of recover after the freezing treatment (ANOVA test: 28.86; p <0.05). Individuals of *P. elegans* required the longest time to recover (56 ± 25 minutes), compared with *P. bogotensis* (Tukey test: 9.26; p<0.05; 4+3 minutes) and with *P. nervicus:* (Tukey test: 9.34; p<0.05; 3+4 minutes). No differences were found in the recovery times between *P. bogotensis* and *P. nervicus* (Tukey test: 0.08; p: 0.99).

Body ice grown during experimental freezing in Paramo frogs

The water content of *Pristimantis* from Paramos was comparable (*P. bogotensis*: 77.04 ± 0.49%; *P. elegans*: 76.62 ± 1.4%; *P. nervicus*: 79.02 ± 2.37%; n = 5 for each species). Whereas *P. bogotensis* and *P. nervicus* presented an exothermic peak when exposed to the experimental freezing protocol, the pattern of body ice formation during the protocol differed between species (Table 1).

Discussion

Cryoprotectant synthesis is the result of adaptation to freezing in temperate frogs (Costanzo and Lee 1993; Costanzo et al. 1993), so it is logical to assume a role for species in other ecological settings involving risk of body freezing. Accordingly, anurans exposed to freezing spells in high-tropical elevations must display such responses or equivalent ones (Navas 1997; Carvajalino-Fernández et al. 2011; Navas et al. 2013). Our study assumed that similar physiological mechanisms exist in such anuran community, and for simplicity focuses on glucose as a first approach to the problem. However, it is likely that molecules like glycerol (Layne 1999) play additional roles, particularly in the context of permanent freeze protection (Moalem et al. 2005; Rexer-Huber et al. 2011), particularly in the most exposed species. Independently of this, our data corroborate the view of glucose as an important and highly flexible molecule associated with freeze tolerance in anurans, including high-elevation tropical lineages. The study also highlights physiological diversity among Paramo anurans and favours the notion of differential impact of freezing spells within the community. Both *P. nervicus* and *P. bogotensis* can be formally defined as freeze tolerant due to glucose spikes

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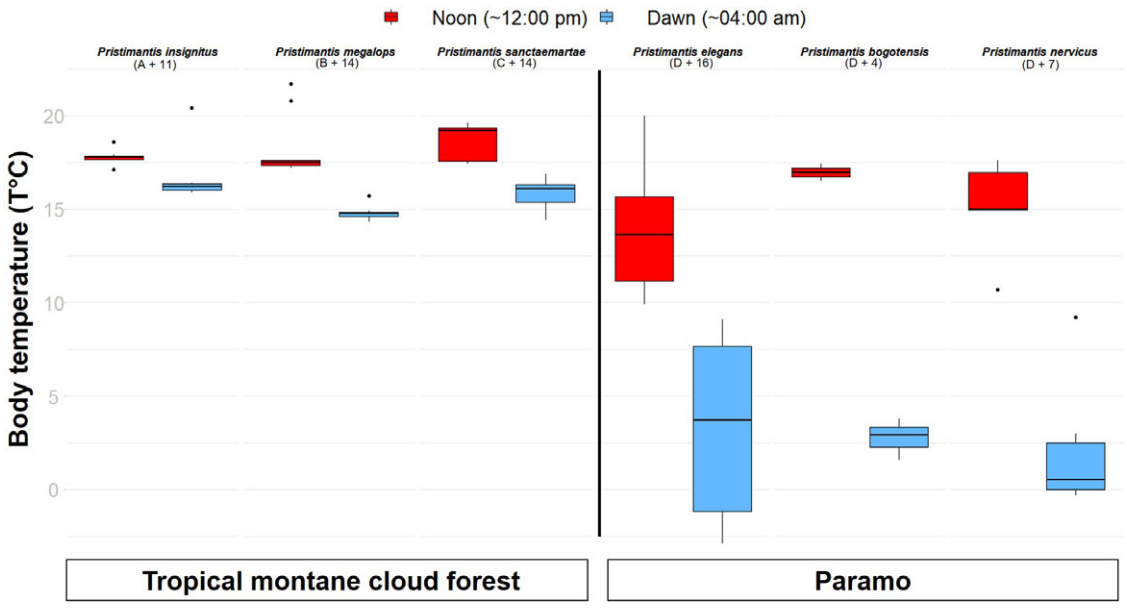

Figure 1. Field body temperature of *Pristimantis* species in terrarium during glucose measures at noon (˜12:00 pm, red boxplot) and dawn (˜4:00 am, blue boxplot). "n" of registers under the name of the species. A, B, C, D, differences of temperatures at dawn between species with letters in each boxplot (using multiple comparisons with Bonferroni correction; significant P≤0.05). +, significant difference between body temperatures at noon and dawn for each species.

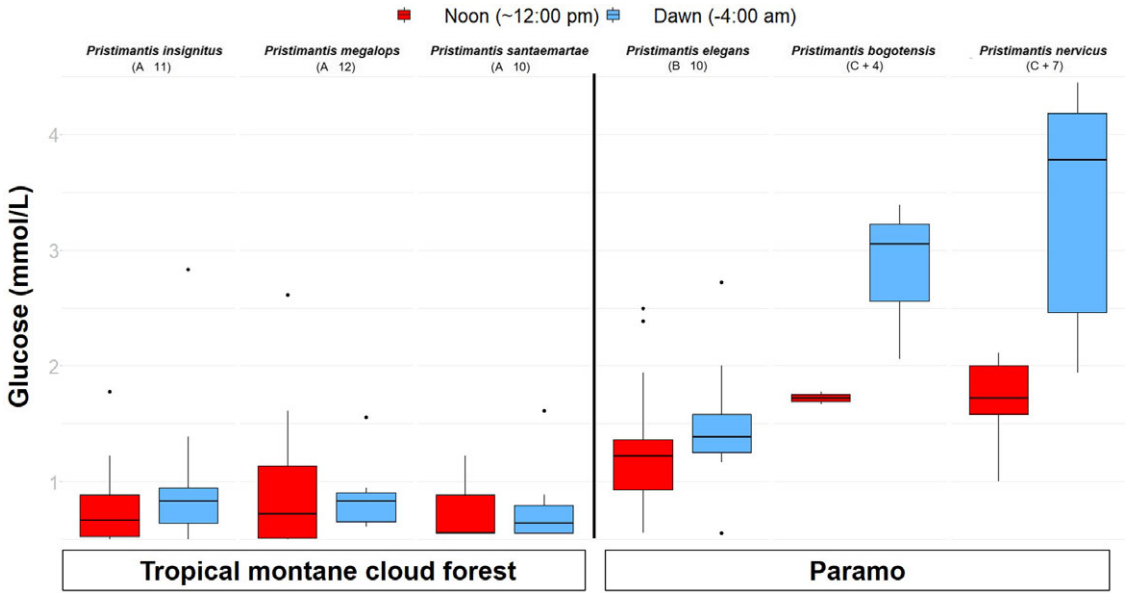

Figure 2. Differences of glucose levels of *Pristimantis* species at noon (˜12:00 pm, red boxplot) and dawn (˜4:00 am, blue boxplot). "n" of registers under the name of the species. A, B, C, D, differences of glucose concentration at dawn between species with letters in each boxplot (using multiple comparisons with Bonferroni correction; significant P≤0.05). +, significant difference between glucose concentration at noon and dawn for each species.

and body ice grow during freeze events (Storey and Storey 1992), but not *P. elegans* because of the absence of body ice formation.

The processes leading to protection may be associated with diel shifts enhancing nocturnal cryoprotection or reactive responses to low temperature, but our data were not designed to tell these two possibilities apart. What we know is that a type of daily glucose modulation is possible and that such modulation is compatible with the highest activity period for the *Pristimantis* in the Paramos during the night, at which time there is a risk of freezing in the Paramo (Navas 1996c; Lüddecke and Sanchez 2002; Navas et al. 2013). Although from one perspective this may seem comparable to freezing in hibernacula, a factor requiring antifreeze adjustment in Alaskan *L. sylvaticus* (Sinclair et al. 2013; Larson et al. 2014; Larson and Barnes, 2016), the issue requires further research because freezing spells occur more often at the time of day of maximum activity. Given that *L. sylvaticus* enters a metabolic depression under conditions interpretable as similar, Paramo and temperate species would converge ecologically only when the latter emerge during the spring.

Despite the above-described parallel between Paramo daily conditions and a temperate spring, it is possible that the freezing ecology of Paramo frogs is closer to that of other lineages which

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Table 1. Reference value of freezing parameters for *Pristimantis elegans*, *P. bogotensis* and *P. nervicus*.

Specie	N	Supercooling point (ºC)	Freezing point (ºC)	Body mass (mg)	Body ice grow (mg)	Body ice (%)	Maximum body ice (%)
Pristimantis bogotensis	8	−3,22±0.20	−2.93±0.15	803.5±143.31	103.83±58.18	17±5.37	63.21
Pristimantis elegans	4	−2.97±0.51	−2.86±0.59	5369.25±1854.34	13,87±11.14	0.34±0.09	0.64
Pristimantis nervicus	5	−2.87±0.09	−1.66±0.66	1333.2±381.57	25.90±12.77	5.67±3.27	11.53

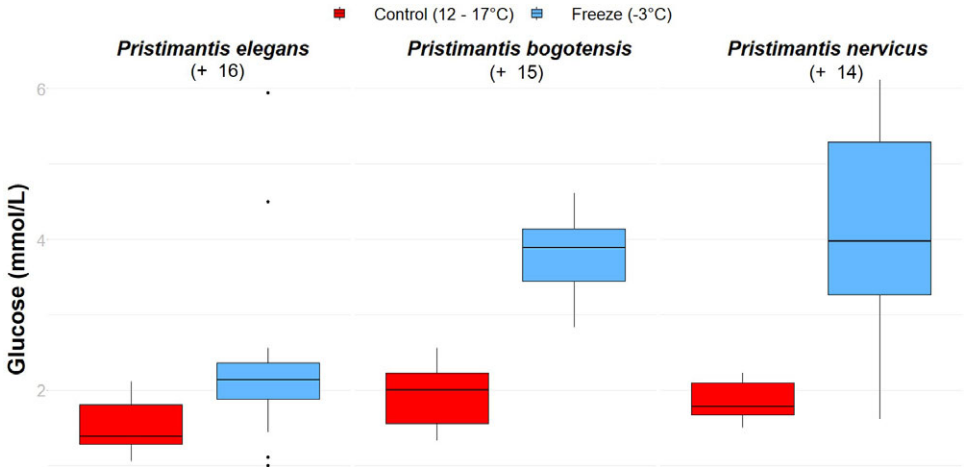

Figure 3. Glucose concentrations changes by experimental freeze exposure in Paramo *Pristimantis* species. For each species, the glucose levels after a freeze exposure of 2 horas of −3 °C were represented by the blue boxplot and the control groups were represented by the red boxplot (for more details see section 2.4). "n" of registers under the name of the species. +, significant difference between glucose measures of freezing treatment and control.

tolerate episodic daily freezing, for example, intertidal invertebrates in temperate zones (for example Farke et al. 1984; Hawes *et al.* 2010; Theede et al. 1976; Waller et al. 2006). These animals survive freezing temperatures during aerial exposure at low tides, although their osmoconformity prevents accumulation of low-mass molecules as cryoprotectants. Instead, they accumulate INPs, perhaps the principal physiological mechanisms for freezing tolerance in marine invertebrates (Hawes et al. 2010; Storey and Storey, 1996). Furthermore, certain insects from cold environments experience daily freezing (Baust and Nishino 1991; Block et al. 1998; Sinclair et al. 1999; Sinclair and Chown 2005), and as *Hyla versicolor* and *Hynobius keyserlingi* do (Layne and Jones 2001; Storey and Storey 1992), they use polyols like glycerol or sorbitol as cryoprotectants; whereas sugars like glucose, maltose and trehalose seem secondary in this context (Storey 1997).

Our conclusions raise several general questions. Given that significant variation in the physiology of freezing occurs among related species within a single genus, even if those species share certain key ecological traits, we anticipate that the overall physiological diversity in the Paramo amphibian community is substantial. This diversity likely involves diel cycles of protection, constant protective baselines and even the scope of reactive protection. The last mentioned would necessarily involve fast mobilisation of cryoprotectants in short order and may possibly be active after a freezing event (Sinclair et al. 2013; Larson et al. 2014). This scenario allows for inferring that climatic shifts leading to changes in the frequency or scope of freezing spells may lead to substantial shift in the structure of high-elevation tropical amphibian communities, mainly around the 3500 m contour. Exposure to freezing spells may last minutes or hours (Navas et al. 2013), so that shifts in this context would make some species less prone to tolerate freeze spells and therefore be limited to lower elevations or less exposed habitats. For example, *P. elegans* may be constrained to thermally buffered habitats such as the vicinity of streams. On a more evolutionary note, our work supports the idea of a physiologically diverse anuran community in which stock from different lineages has adapted to Paramos acquiring physiological characteristics not present in lower elevation counterparts.

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