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ABSTRACT: Phenotypic variation along environmental gradients—particularly in body size—occurs in a variety of species. Larger-bodied individuals are usually found in colder climates, as predicted by Bergmann's rule. In ectotherms, this pattern remains controversial. Among thermoconformers, smaller body sizes are expected in colder climates because these species might have relatively shorter warm-up times (advantageous in cold climates), whereas the reverse pattern can be expected in thermoregulators (heat-balance hypothesis). In amphibians, additional factors like humidity and thermal niche might also contribute to body-size variation. Following Allen's rule, there can also be a negative relationship between temperature and relative limb length. Here, we described associations among temperature, precipitation, body size, and relative limb length in *Calotriton asper*. We expected individuals from higher elevations (colder climates) to be smaller when compared to low-elevation conspecifics. We found an influence of temperature on body-size variation but, contrary to expectations, salamanders from colder climates were larger compared to low-elevation populations, which corroborates with the heat-balance hypothesis. In accordance with the converse water-availability hypothesis, we also demonstrated that precipitation was related to body-size variation in this species. Finally, our results supported the predictions of Allen's rule. This trend could be the result of evolutionary responses to harsh environments, driven by either local adaptation, plasticity processes, or a combination of both.

Key words: Allen's rule; Amphibian; Bergmann's rule; Elevation gradient; Leg length variation; Morphological variation

ENVIRONMENTAL CONDITIONS—and temperature in particular—vary with geographical gradients such as latitude (Sunday et al. 2014) or elevation (Zamora-Camacho et al. 2015), but are also bound to weather, seasonality, or habitat structure (Hammerson 1989; Ortega et al. 2014). Ectothermic organisms are sensitive to temperature variation, as most of their bodily functions depend on environmental (operative) temperature (Angilletta et al. 2002). For instance, temperature variation can constrain thermoregulation or mobility (Carroll et al. 2009; Sinervo et al. 2010), potentially driving local extinctions (Dunham 1993; Buckley et al. 2010). Such variation can also be linked to differences in individual-level traits, such as ecological performance, physiology, behavior, or life-history traits (Billerbeck et al. 2001; Huey and Berrigan 2001; Cano and Nicieza 2006; Navas et al. 2008).

Evidence for phenotypic divergence along environmental gradients is available from a variety of species (Morrison and Hero 2003; Keller et al. 2013). Notably, body size varies within species, with larger-bodied individuals usually found in cold climates (i.e., high-elevation and high-latitude sites). This trend follows Bergmann's rule, originally conceived for endothermic vertebrates (Bergmann 1847; Mayr 1956). The most commonly invoked explanation underlying this pattern is the heat-conservation hypothesis. This hypothesis posits that, for a given shape, larger animals have a lower surface area–volume ratio (SVR) than smaller individuals, which limits body-heat loss and promotes thermal inertia. In turn,

this reduces the cost of body temperature maintenance in cold conditions (Walters and Hassall 2006). Consequently, relatively larger individuals are expected to occur in colder environments, as a response to both elevational and latitudinal shifts in temperature. A trend for larger body size in colder habitats might be considered as an adaptive response to minimize heat loss (Olalla-Tárraga and Rodriguez 2007).

Ectothermic vertebrates depend mainly on external heat sources and body temperature reflects ambient temperatures. Some species employ behavioral thermoregulation mechanisms to achieve optimal body temperature (thermoregulators; Carrascal et al. 1992; Hutchison and Dupré 1992; Belliure and Carrascal 2002). Other species have limited thermoregulatory opportunities (thermoconformers; Hadamová and Gvoždík 2011). Although attempts have been made to apply Bergmann's rule to ectotherms, no clear pattern has emerged (Mousseau 1997; Ashton and Feldman 2003; Olalla-Tárraga and Rodriguez 2007; Adams and Church 2008; Rivas et al. 2018).

The heat balance hypothesis was proposed as an expansion of Bergmann's rule to explain patterns in ectotherms that actively thermoregulate (Olalla-Tárraga and Rodríguez 2007). From this hypothesis, larger body sizes are expected in colder climates because they provide advantages during thermoregulation (lower SVR are associated with greater capacity for heat conservation; Olalla-Tárraga and Rodríguez 2007). However, the hypothesis might not apply to thermoconforming species (Blackburn et al. 1999; Olalla-Tárraga and Rodríguez 2007) which passively

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follow the available environmental temperatures (Hadamova and Gvozdik 2011). Among thermoconformers, smaller body sizes could be favored in colder climates because a higher SVR might provide relatively shorter heating times, leading to a reverse Bergmann cline (Olalla-Tárraga and Rodriguez 2007).

For reasons similar to those explaining a reverse Bergmann's rule among thermoconformers, anatomical extremities are also expected to be relatively shorter in animals occurring colder climates when compared to conspecifics in warmer conditions (cf. Allen's rule; Ray 1960; Reiss 1989). Among ectothermic species, empirical studies offer little support for this hypothesis (but see Bidau and Marti 2008; Langkilde 2009). Investigating changes in morphology along environmental gradients in ectotherms is important to improve our understanding of the interplay between elevation, temperature, precipitation, body size, and leg length.

Amphibians are typically thermoconformers (e.g., Olalla-Tárraga and Rodríguez 2007; Ficetola et al. 2010), and are dependent on moist conditions because of their permeable skin—and associated risk of desiccation—and the fact that the larval life-history is usually aquatic. These characteristics could explain why a unified clinal pattern among amphibian species is still lacking, even if various explanations for the body-size variation along ecogeographical gradient are likely (e.g., Adams and Church 2008). For instance, a large body size in amphibians might be advantageous in either (1) dry climates, because a lower SVR confers greater resistance to desiccation (negative relationship between body size and precipitation, as expected under the water-availability hypothesis; Ashton 2002; Gouveia and Correia 2016); or (2) wet areas, because the activity of amphibians is often associated with moist conditions (positive relationship between body size and precipitation, as expected under the converse water-availability hypothesis; Zug et al. 2001; Ficetola et al. 2010; Valenzuela-Sánchez et al. 2015).

Rypel (2014) suggested that understanding the thermal niche of a species could facilitate a prediction of whether or not its body-size variation follows Bergmann's rule, or its inverse. Specifically, cool-water dependent species should demonstrate Bergmann's rule (i.e., larger individuals in cold climates), whereas species depending on warm waters might show the reverse pattern (Rypel 2014). Moreover, larger females living at higher elevations might produce fewer but larger eggs (an adaptive strategy adopted to improve offspring survival in harsh environments; Morrison and Hero 2003), leading to larger metamorphs and larger adults (Voss 1993; Liao and Lu 2012). Because these mechanisms are not mutually exclusive, it is difficult to tease apart individual contributions to body-size variation. Therefore, many environmental and ecological variables can interact and lead to body-size variation along ecogeographical clines, driven by different mechanisms (Stillwell 2010). As a result, patterns consistent with Bergmann's rule are apparent in some amphibian species (Miaud et al. 2000; Adams and Church 2008; Ficetola et al. 2010; Liao and Lu 2012), but not others (Morrison and Hero 2002; Krizmanic et al. 2005; Laugen et al. 2005; Liao et al. 2010). In the latter cases, thermoregulatory opportunities, habitat quality, predation pressure, or incomplete sampling of the elevational gradients were among the reasons invoked to account for exceptions to

the rule (Howard and Wallace 1985; Ryser 1996; Morrison and Hero 2002; Cvetkovic et al. 2009). In this context, further research on the relationships between abiotic factors and body sizes among amphibian species is needed.

Here, we investigate how temperature and precipitation might influence phenotypic variability in Pyrenean Newts (*Calotriton asper*), an endemic and protected salamander of the Pyrenees Mountains. As with many amphibians, this species is a poor disperser (Smith and Green 2005; Milà et al. 2010), which can favor local adaptation (by limiting gene flow) and high phenotypic variability along environmental gradients (Clergue-Gazeau and Martinez-Rica 1978; Clergue-Gazeau and Bonnet 1980; Serra-Cobo et al. 2000). Little is known about thermoregulation among salamander species. They are commonly defined as thermoconformers, despite studies suggesting that some species could have the capacity for behavioral thermoregulation through the selection of particular microhabitats (e.g., Heath 1975; Balogová and Gvoždík 2015). To improve understanding of the effects of high elevation and the associated environmental variables on amphibian body size, we sampled Pyrenean Newts from a range of elevations. As would be expected for thermoconforming ectotherms, we predicted a reverse Bergmann gradient (with higher elevations occupied by smaller individuals when compared to low-elevation sites) because a higher SVR might have relatively shorter warm-up time, which could be advantageous in cold climates. For similar thermoregulatory reasons, we also expected individuals from higher elevations to have relatively smaller hind limbs (positive relationship between hind-limb length and temperature), as expected under Allen's rule. Then, we predicted that individuals would be smaller in wetter habitats (i.e., higher elevations), as expected under the water-availability hypothesis.

Materials and Methods
Study Species

Pyrenean Newts (*Calotriton asper*) are large-bodied urodeles occurring in brooks, lakes, springs, and aquatic caves, from 250 to 2500 m (Martinez-Rica and Clergue-Gazeau 1977; Clergue-Gazeau and Martinez-Rica 1978). Previous work has reported divergences in life cycle, morphology, and color pattern as a function of site elevation (Martinez-Rica 1980; Gasser and Clergue-Gazeau 1981; Serra-Cobo et al. 2000; Oromi et al. 2014). At 300 m, the activity season spans from May through September, whereas populations at 2500 m have activity limited to July–September. At low-elevation sites, some individuals enter a terrestrial life history in summer months, whereas individuals at high-elevation sites are aquatic throughout the year. Pyrenean Newts exhibit sexual size dimorphism, with males being larger than females (Guerrero-Campo 1995; Serra-Cobo et al. 2000). The species is listed on Appendix II of the Bern Convention, Annex IV of the EU Habitats Directive, and as near threatened by the IUCN (Bosch et al. 2009).

Sampling Sites and Morphological Measurements

Sampling sites were selected based on presence–absence data available from naturalist associations and scientific societies, and at which high population densities had been previously detected. Subjects were collected by hand from

mountain streams during the 2017 breeding season (10 June–27 July). To minimize bias in our analyses, and because juveniles are less common and more delicate, we collected only adult subjects. In total, we sampled 159 adults from 8 different sites ($n = 99$ individuals from 5 Spanish populations, and $n = 60$ individuals from 3 French populations; Fig. 1), from 625 to 2189 m in elevation (Table 1; all values for site elevation are reported as meters above sea level). For each individual, we recorded sex and measured the snout–vent length (SVL; taken from the tip of the snout to the cloacal opening) and the left hind-limb length (HLL) with calipers (±0.1 mm). Subjects were then immediately released at their capture sites.

Climatic Variables

Based on CHELSA data (Karger et al. 2017), we calculated mean values for annual precipitation and air temperature from 1979–2013 at each of the sampled sites. Such data are a downscaled model output of temperature and precipitation estimates of the ERA-Interim climatic reanalysis (Dee et al. 2011) to a high spatial resolution of 30 arcsec (~1 km^2). The model had been specifically developed to investigate ecological issues according to climate gradient, such as Bergmann's rule. Air temperature is a good proxy for water temperature (e.g., Caissie 2006; see Fig. S1 in the Supplementary Materials available online). Indeed, air and water temperatures are positively correlated, except in circumstances where water temperatures are impacted by underground water sources or when air temperatures reach colder values (e.g., winter). Air and water temperatures are closely correlated at our collection sites because we focused on: (1) a highland area where underground water has little impact on the river water regime, and (2) mean yearly temperature. Further, we used mean temperature rather than minimum or maximum temperatures because we sampled populations at the elevation extremes of their distribution (mean temperature integrates minimal and maximal temperatures). Also, results using minimum temperatures were similar to those using mean temperatures (personal observations). Temperature values along the elevation gradient encompassed by our sampling sites varied from 2.5°C (Néouvielle at 2189 m) to 11.8°C (Hostalets d'en Bas at 625 m; see Fig. S2 in the Supplementary Materials available online).

Statistical Analyses

We investigated the relationship between adult SVL and temperature with the use of a linear regression model with SVL as response variable (following a Gaussian distribution) and temperature, sexes, and the first-order interaction between both variables as explanatory variables. A similar model was then fitted where the temperature effect was replaced by a precipitation effect among the set of explanatory variables. We tested the temperature and precipitation effects in separate models because the high correlation between them (Spearman correlation = −0.78, $P < 0.001$) can contribute to multicollinearity in a complete model. Multicollinearity among explanatory variables inflates the variance of regression parameters, which potentially leads to both unstable model fit (attributable to high variance) and error in identification of relevant predictors, as well as in their relative importance assessment (e.g.,

Dormann et al. 2013). We also examined the correlation between temperature and leg length. Because HLL was strongly correlated with SVL (Spearman correlation = 0.73, $P < 0.001$), we included SVL as covariate to compare size-corrected hind-leg length. A second generalized linear model was then built with HLL used as a response variable (following a Poisson distribution) and temperature, sex, and the first-order interactions with SVL as explanatory variables. Response and explanatory variables were scaled for comparison purposes. Model selection was performed with backward selection. Nonsignificant interactions were first removed, followed by nonsignificant main effects in reduced models. Between each step, successive models were compared using likelihood-ratio tests (LRT) to determine the significance of the variable removed, as recommended by Burnham and Anderson (2002). If the effect of this variable was not significant ($P > 0.05$), the new model was kept and the backward selection was continued. The procedure was stopped when all explanatory variables had a significant effect on the response variable. Models were performed with the use of R v2.14.2 (R Development Core Team 2011).

Results

We found strong variation in adult body size across the eight studied populations. Mean (±1 SD) SVL ranged from 68.2 ± 0.8 mm in low-elevation Pyrenean salamanders (from the Hostalets d'en Bas population, 625 m) to 73.6 ± 1.2 mm at the higher-elevation population (from the Néouvielle population, 2189 m). Adult body-size variation did not vary between sexes, and was correlated with temperature with larger individuals found at higher elevations (LRT: $F_{1,156} = 55.32$, $P < 0.001$, $R^2 = 0.26$; Table 2; Fig. 2a). Mean SVL was also related to precipitation with larger individuals found at higher elevations (LRT: $F_{1,156} = 104.6$, $P < 0.001$, $R^2 = 0.40$; Table 2, Fig. 2b).

Individuals from the lowest elevation site had legs with a mean length of 18.6 ± 0.5 mm (relative HLL [HLL/SVL ratio] = 0.273 ± 0.006), whereas conspecifics from the highest-elevation site had longer legs (HLL = 22.9 ± 0.3 mm; relative HLL = 0.312 ± 0.004). After accounting for variation in body size, temperature and HLL were negatively correlated (LRT: $\chi^2 = -12.39$, df = 1, $P < 0.001$; Table 2; Fig. 2c). Sex did not influence relative HLL.

Discussion

Our data improve the understanding of the relationships between temperature and precipitation on body-size variation in a thermoconforming amphibian. We found that adult Pyrenean Newts from higher elevations (colder, wetter climates) were larger, with relatively longer legs compared to low-elevation populations, and that this pattern was consistent in both sexes. This result corroborates evidence from other studies documenting Bergmann's rule among salamanders, where larger individuals occur in colder climates (e.g., *Cynops pyrrhogaster* [Marunouchin et al. 2000], *Lissotriton vulgaris* [Sotiropoulos et al. 2008], *Triturus karelinii* [Üzüm and Olgun 2009], *Triturus carnifex* [Ficetola et al. 2010], and four plethodontid species [Peterman et al. 2016]). We also documented a positive relationship between body size and precipitation, a result that contrasts with the converse water-availability hypothesis,
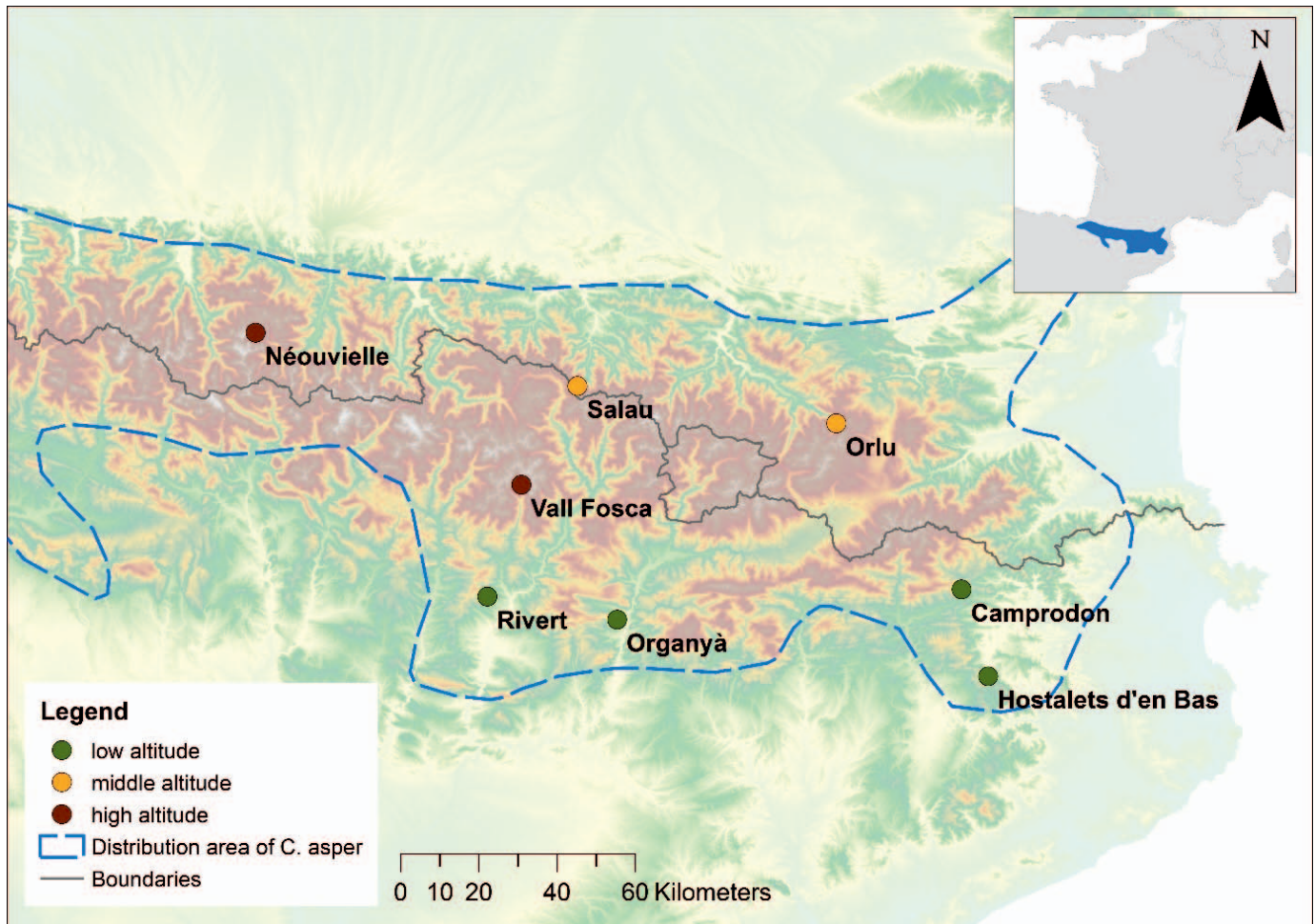
Fig. 1.—Map of the study area (France, Andorra, and Spain) showing the eight *Calotriton asper* populations sampled in 2017. The highlighted area within the inset map shows the known distribution of the species.

assuming that larger body sizes occurring in humid conditions (i.e., high-elevation sites) favors the activity of those individuals (Zug et al. 2001). Indeed, moist conditions can increase the availability of certain prey types (e.g., worms), which influences the foraging activity among some amphibian species (e.g., Feder and Burggren 1992). Also, because humid conditions can minimize the risk of desiccation in amphibians, the distances traveled by individuals should increase when moisture levels are high (Trochet et al. 2017).

Our findings did not support the hypothesis that smaller body size is favored under cold climates because a higher

SVR provides relatively shorter warming times (Olalla-Tárraga and Rodriguez 2007). Instead, larger bodies might be advantageous at high elevations for several nonexclusive reasons (Blackburn et al. 1999; Ficetola et al. 2010). First, larger body size might provide advantages during thermo-regulation because a lower SVR would help conserve body heat (Bergmann 1847; Walters and Hassall 2006; Ficetola et al. 2010). Second, large-bodied female amphibians living at high elevation might produce fewer but larger eggs, because the allocation of more energy into each offspring at high elevations is considered an adaptive strategy adopted to improve survival of their offspring in harsh environments

TABLE 1.—Locality and climate data for populations of *Calotriton asper* occurring in France and Spain that were sampled in 2017. Geographical coordinates are based on WGS84 datum, elevation is reported as meters above sea level, and other values are reported as means ± 1 SD.

Population	Number of adult subjects (males; females)	Coordinates (latitude/longitude)	Elevation (m)	Temperature (°C)	Annual precipitation (mm)
Hostalets d'en Bas	20 (9 male; 11 female)	42.085755°N, 2.445868°E	625	11.80 ± 11.95	794 ± 66.22
Organyà	20 (13 male; 7 female)	42.203893°N, 1.297851°E	773	10.89 ± 10.96	591 ± 49.31
Rivert	20 (6 male; 14 female)	42.249220°N, 0.895344°E	893	10.63 ± 10.72	663 ± 55.36
Camprodon	20 (8 male; 12 female)	42.284526°N, 2.361725°E	979	10.25 ± 10.36	679 ± 56.68
Salau	20 (7 male; 13 female)	42.738443°N, 1.158159°E	1258	6.72 ± 7.86	868 ± 73.30
Vall Fosca	19 (7 male; 12 female)	42.508657°N, 0.991194°E	1828	6.55 ± 7.81	947 ± 80.37
Orlu	20 (10 male; 10 female)	42.663393°N, 1.967250°E	1313	3.00 ± 4.11	830 ± 71.00
Néouvielle	20 (13 male; 7 female)	42.839112°N, 0.148971°E	2189	2.45 ± 5.40	1017 ± 84.51

TABLE 2.—Summary of generalized linear models showing the relationships between abiotic and morphometric variables (snout–vent length = SVL; hind-limb length = HLL) among adult Pyrenean Newts (*Calotriton asper*) sampled from eight populations in 2017. Variables were retained in the models with the use of a reverse selection process; values for relative HLL were derived from residuals of a linear regression between HLL and SVL.

Model		Variables	Estimated coefficients[a]
1. SVL and temperature	Main effects	Intercept	1.1751*
		Temperature	−0.1498*
		Sex	Not retained
	Interaction	Sex:Temperature	Not retained
2. SVL and precipitation	Main effects	Intercept	−3.6433*
		Precipitation	0.0046*
		Sex	Not retained
	Interaction	Sex:Precipitation	Not retained
3. Relative HLL and temperature	Main effects	Intercept	2.4495*
		Temperature	−0.0212*
		Sex	Not retained
		SVL (covariate)	0.0106*
	Interaction	Sex:Temperature	Not retained

[a] Asterisks indicate $P < 0.001$.

(Morrison and Hero 2003). In turn, this strategy produces larger metamorphs and adults (Voss 1993; Liao and Lu 2012). Indeed, allocating limited resources into larger juveniles might allow them to increase survival rates in colder environments (because lower SVR could confer a higher capacity for heat conservation; Morrison and Hero 2003; Dziminski and Roberts 2006). Synergistically, cold temperatures can also extend development rates in amphibian species (Atkinson 1994), which in turn results in larger body size in metamorphosed individuals and adults (Miaud et al. 1999; Ficetola and De Bernardi 2006). Finally, our results corroborate Rypel's (2014) hypothesis, wherein cool-water dependent species are expected to follow Bergmann's rule. Indeed, Pyrenean Newts were larger at higher-elevation sites, and are dependent on cool and well-oxygenated waters (Rypel 2014; Rollinson and Rowe 2018). It is worth noting that adult body size in amphibians might also be affected by processes other than temperature that also vary along an elevational gradient. At high elevations,

amphibians tend to have shorter activity periods and greater longevity than their low-elevation conspecifics (Morrison and Hero 2003; Iturra-Cid et al. 2010; Oromi et al. 2012, 2014). In turn, this pattern might influence the adult body size attained. Also, metabolic rate or predation pressure can vary along an elevational gradient, both of which might affect adult body size (Diego-Rasilla 2003; Zhang and Lu 2012; Dupoué et al. 2017).

Hind-limb length (relative to body size) varied along the elevational gradient in Pyrenean Newts. Although several studies showed the opposite pattern (Woodruff 1975; Krasnov et al. 1996; Iraeta et al. 2011), we found a negative relationship between relative HLL (corrected for body size) and temperature (see Kubišová et al. 2007; Zamora-Camacho et al. 2015) as expected under Allen's rule. At high elevations, larger legs could compensate for high-elevation constraints (reduced diel and seasonal activity), which would select for higher locomotory abilities that help maintain connectivity between populations (in spite of a short activity period). Genetic analyses are needed to test this hypothesis. Secondly, relatively longer legs might also enhance spermatophore transfer during courtship at high elevation, where fecundity is typically lower among the amphibian species occurring there (Morrison and Hero 2003). For example, Crested Newts (*Triturus cristatus*) having elongated limbs improve the spermatophore maneuver during courtship, which, in turn, increases breeding success (Rehák 1983; Kubišová et al. 2007).

In conclusion, our findings collectively demonstrated a relevant Bergmann's cline in Pyrenean Newts. Even in primarily aquatic species, measures of air temperature provide a reliable environmental proxy for temperatures experienced in the water (e.g., Caissie 2006; Fig. S1). We have shown that adult individuals were larger in cooler and wetter climates, consistent with the heat-balance hypothesis and the converse water-availability hypothesis. Additionally, individuals from higher elevations had longer hind limbs compared to those from low-elevation sites. Larger body size in colder climates (i.e., higher elevation) could stem from responses to harsh environments, driven by either local
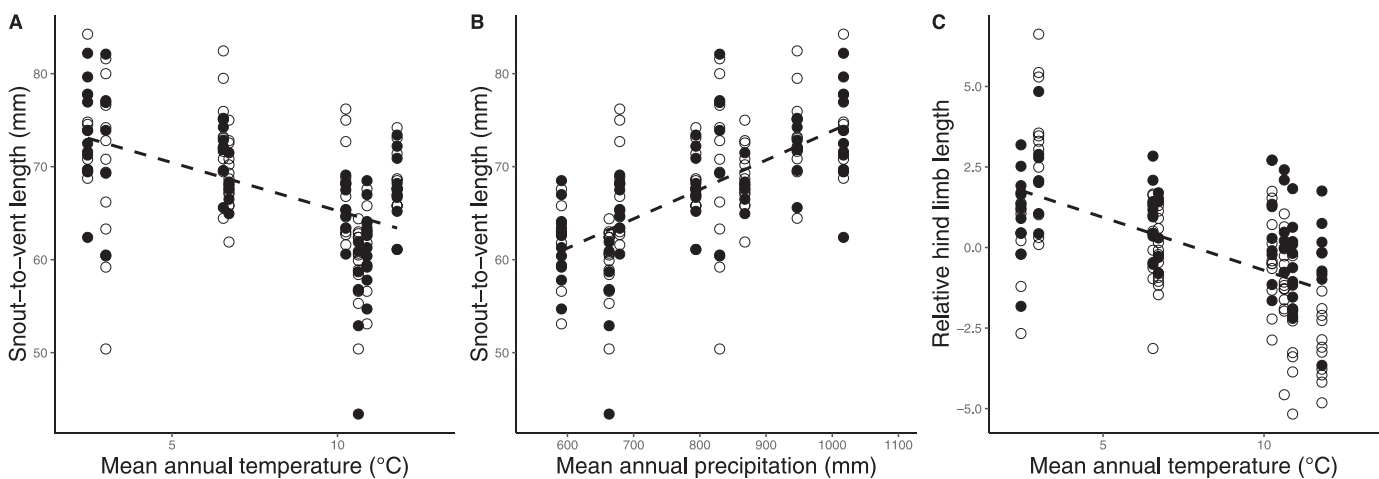

FIG. 2.—Relationships between abiotic variables and morphometric measurements of adult Pyrenean Newts (*Calotriton asper*) sampled in 2017. (A) Mean annual temperature (°C) vs. snout–vent length (SVL, in mm); (B) mean annual precipitation (mm) vs. SVL; (C) mean annual temperature vs. relative hind-limb length (derived from residuals of the linear regression between hind-limb length and SVL). Filled circles = males; open circles = females. Dotted lines represent the regression lines based on linear models.

adaptation, plasticity, or both. Complementary studies are still needed to support our interpretations and improve our understanding on the influence of elevation, temperature, and precipitation on mechanisms underlying body-size variation along an elevation gradient in ectotherms (Stillwell 2010). Such studies should be framed in the context of predicting the future of *C. asper* populations under different scenarios of climate change. Indeed, morphological variation, particularly a reduction in body size that could accelerate rates of heat exchange, are expected responses in the face of global warming (Millien et al. 2006; Gardner et al. 2011). Such environmental changes can modify thermal constraints along the altitudinal gradient, with possible consequences on thermoregulation processes, locomotion, individual fitness and survival in ectotherms, and subsequently population viability (Navas et al. 2008).

SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at https://doi.org/Herpetologica-D-18-00011.S1

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