Plastic responses to temperature vs. local adaptation at the cold extreme of the climate gradient

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Abstract Climate is a strong selection agent at high elevations, but experimental examinations of how animals exclusive of highlands cope with its variation are scarce. We analysed temperature-induced variation of early ontogenetic traits in the alpine grasshopper *Chorthippus cazurroi*, and compared populations from the elevational extremes of the species distribution under laboratory conditions spanning natural temperature ranges. Neither elevation of origin, nor different growing temperatures, had a direct effect on nymph body size, but both factors contributed to size at hatching indirectly, via their effect on the duration of embryo development. Large emerging nymphs had a consistently greater survival, although small and fast-developing nymphs from highlands also performed well at low temperatures. Viability selection favoured fast-developing phenotypes in conditions in which plasticity delayed development, in a typical countergradient pattern. Growth in the successive stage did not compensate for slow development at hatching, thus responses at this early stage have potential long-lasting consequences. Although phenotypic selection during early development certifies the strength of selection imposed by cold temperatures in the laboratory, elevation clines of body size did not emerge in either nymphs or the wild parental generation. Differentiation in the wild may be levelled out by fecundity selection for large sizes, drift and gene flow resulting from the fragmentation and proximity of populations, or by micro-climatic differences that reduce the likelihood of directional selection. There is therefore potential for local adaptation to temperature, but a series of conditions typical of alpine environments and ectotherms may impair, confound or constrain full differentiation along the gradient.

Keywords Alpine fauna, body size, countergradient selection, development, phenotypic selection, selection gradients

Introduction

Climate is one of the major agents of natural selection and organisms have developed a wide-array of adaptations to cope with it, especially in physiological, morphological or phenological characteristics associated with growth and development (Johnston and Bennett 1996; Angiletta et al. 2004). These characteristics also display high levels of phenotypic plasticity, although most often in combination with genotypic variation, since plastic genotypes tend to be poorly efficient in coping with extreme conditions and developing as extreme phenotypes compared to locally adapted genotypes (DeWitt et al. 1998; Santamaria et al. 2003) (Fig. 1b). Local adaptations mainly result from non-additive gene-environment interactions: genotypes differ in their sensitivity to the environment (which we refer to as climate) and the performance of a given genotype depends on specific climatic conditions (Kawecki and Ebert 2004; Törang et al. 2015) (Fig. 1c). In nature, gene-environment interactions often underlie clinal phenotypic variation, such as ecotypic differentiation in body size and shape (Barton 1999; Santos et al. 2004).

Phenotypic variation may be further enhanced, or drastically reduced, by gene-environment covariation associated with the non-random distribution of genotypes across climate regions. If we consider growth patterns, slow-growing genotypes may be favoured at high latitudes or elevations, where the environment promotes slow growth because low temperatures decelerate metabolic and physiological activities (Arendt 1997). This alignment of genetic and environmental influences on phenotypic expression produces positive covariation, or cogradient variation, which tends to accentuate phenotypic variation across the gradient (Levinton 1983) (Fig. 1d). Cogradient clines have been described most often in plants (Aarssen and Clauss 1992;

Lusk et al. 2008), especially in mountains, where genotypes for small plant size are found primarily on uplands where also the environment hampers somatic growth (Körner 2003). Alternatively, strong time constraints may challenge organisms and select for genetically rapid growth and development with increasing latitude and elevation to compensate for environmental conditions that slow down these processes (Berven and Gill 1983; Conover and Schultz 1995). This produces counter-gradient variation and negative genotype-environment covariation: opposing genetic and environmental influences reduce the magnitude of diversification along the gradient (Conover et al. 2009) (Fig. 1e). Many of the known cases of coutergradient variation involve traits associated with vital rates in poikilotherms (metabolism, growth, development) and temperature-dependent sex determination mechanisms (Conover and Heins 1987; Atkinson 1995; Arendt 1997).

Local adaptations also result from gene-environment covariation (Kawecki and Ebert 2004). Countergradient selection on traits counteracts plasticity, which is often a maladaptive by-product of thermal influences on physiology, and local adaptations are manifested as reductions in phenotypic variation among populations across the climate gradient (Conover and Schultz 1995). Plasticity in this case is unable to produce the favoured phenotype or to follow the course favoured by directional selection. On the contrary, cogradient variation yields adaptive-plasticity because the reaction norm is pushed by the environment in the same direction of adaptive genetic change (Ghalambor et al. 2007). Phenotypic selection on traits, and the nature of plastic response, become especially relevant in adjacent populations at latitudinal or altitudinal range limits, or in facing natural or anthropogenic fluctuating climatic conditions, since novel conditions are close enough (in space or in time) to trigger

evolutionary responses and shape the dynamics of populations (e.g. extinction-colonization processes) (Byers et al. 2004).

In this study, we analyse temperature-induced variation in early ontogenetic traits in the alpine grasshopper *Chorthippus cazurroi* (Orthoptera: Acrididae Gomphicerinae), and test whether responses during early life are linked to survival components of fitness. We compared four close local populations (< 20 km) from the elevational extremes of the species distribution under common laboratory conditions spanning natural temperature ranges. We focused on a series of early ontogenetic traits regarding development and body size of embryos and early instars. These life-history stages depend more on temperature alone than older stages in which nutritional conditions, thermoregulation behaviour and sexual differences also matter (Samietz et al. 2005; Berner and Blanckenhorn 2006; Parsons and Joern 2014; Rotvit and Jacobsen 2014). Body size is one of the phenotypic traits that most often changes along climatic gradients, often plastically but also displaying gene-environment interactions, or covariation (Angiletta et al. 2004; Byers et al. 2004). Theory and most empirical studies assume a positive relationship between size at hatching and survival, fecundity and growth at later life stages (Roff 2002; Taylor et al. 1998). In many ectotherms with annual life cycles, however, body size trades off with development time, and countergradient variation permits individuals of colder climate to mature and breed in a short season, although at smaller sizes (Telfer and Hassall 1999; Berner et al. 2004; Byers et al. 2004; Parsons and Joern 2014). In spite of the above knowledge, it remains largely and empirically unresolved whether negative selection on development time is sufficient to oppose positive direct selection on body size in animal populations (Kingsolver and Huey 2008). We analyse (1) the variation of early nymph body size and development with respect to elevation at three temperature

treatments, and (2) the selection gradients imposed by temperature on body size and development time at these early stages of life. If countergradient selection occurs, we expect temperature to accelerate growth, but fast-growing phenotypes occurring in colder climates to counteract environmental (plastic) responses (Parsons and Joern 2014). Moreover, the characteristics at hatching that become important for nymph survival should differ among thermal conditions (Altwegg and Reyer 2003), and local adaptations in the form of (countergradient) fast phenotypes should be favoured at low temperatures, and slow ones at warm temperatures. The opposite is expected if cogradient variation occurs (Arendt 1997).

Methods

Study species and populations

Chorthippus cazurroi is an annual grasshopper endemic to Cantabrian Mountains, north-western Spain. Its geographical range is restricted to an area of approximately 380 km^2 and almost exclusively confined to the alpine belt (Laiolo et al. 2013; 2015). The study was performed in two massifs hosting the widest elevation range of this grasshopper, the Western and Eastern Massifs of Picos de Europa, the maximum height of which reaches 2596 and 2444 m a.s.l., respectively. We centred on two small low-elevation populations, Vegarredonda at 1465 m a.s.l. and Casetón de Ándara (hereafter Ándara) at 1650 m, and two more abundant ones in highlands, Traviesos at 2350 m and Rasa de la Inagotable (hereafter Rasa) at 2180 m (Table S1). The distance between low and high elevation sites within the same massif is 3.8 km (Vegarredonda –Traviesos in the Western Massif) and 1.7 km (Ándara – Rasa in the Eastern Massif),

while that between the two massifs is approximately 20 km. In the first days of September 2012, we collected adults from the four sites, obtaining 10-13 females and 5-9 males from lowland populations, and 25-26 females and 12-13 males from highland populations. These individuals provided eggs for the first generation offspring for the experiment.

Grasshopper rearing

The wild-caught individuals from each collection site were kept under natural photoperiod and temperature (25-30 ºC day /19-22 ºC night) conditions, and maintained in a group in 12 x 25 x 40 cm plastic jars with a perforated cap and a 3 cm layer of moist sand. Each day we provided field cut grasses and sedges (*Brachypodium*, *Poa, Festuca* and *Carex* spp.) and replaced the sand while checking for egg pods. Individuals reproduced during 10-15 days and at death their hind femur was measured as an indicator of body size using a stereo LEICA M125 fitted with an ocular micrometer (accuracy 0.1 mm) (Laiolo et al. 2013). It was not possible to assign the egg pod to a single female since adults were kept in a group, but we assume that most females laid eggs in roughly equal numbers due to the long clutch-laying lags (3-7 days; personal observations). Overall we obtained ≥ 30 egg pods from each local population with the exception of the Vegarredonda population, in which only 16 were laid. Egg pods were maintained at room temperature (conditions as above) in moist filter paper for 30 days, and when necessary (2 cases) they were treated with a mixture of fungicide (0.1% sulphanilamide) and bacteriocide (0.1% methyl-p-hydroxybenzoate) following vanWingerden et al. (1991). The inclusion or exclusion of these pods produce similar results, thus we present results obtained with the complete

data set. Subsequently, egg pods were placed in 1.5 ml plastic tubes and transferred to a refrigerator at 5ºC for 4-4.5 months to break diapause.

Eggs were incubated and grasshoppers reared in incubator chambers with a transparent front door (size: 42 x 34 x 48 cm; Lucky Reptile Herp Nursery II). In order to reduce temperature gradients inside the device and maintain oscillations within 1ºC from the set temperatures (see below), temperature was controlled by digital thermostats (Lucky Reptile Thermo Control PRO II) and only the central shelter was used to place rearing containers. The day/night temperature and photoperiod were set at cycles of 13/11 hours. A 50-W electric lamp was located in front of each chamber and interior lighting was also turned on to provide artificial lightening in both visible and UV spectra during the light time. Egg pods were incubated individually in polystyrene jars (4 cm diameter x 6.5 cm height) topped with a thin sieve mesh and filled with 0.5 cm of moist sand (10% water). Jars were checked every day for hatched nymphs, and after eight days had passed since the last egg hatched in a chamber, pods were removed from chambers.

Hatched nymphs were grown in individual jars as those used for eggs, which were filled with a thin layer of moist sand that was replaced every third day. Nymphs were fed daily with the same mixture provided to adults plus wheat leaves grown in greenhouses to increment protein input. During the day we maintained the sand and the grass moist, moistening most often at high temperatures. In incubator chambers humidity averaged 68.26% ± 0.45 SE and its fluctuations were recorded over a period of five days by means of HOBO sensors installed in chambers at different temperatures (see below). We did not highlight significant differences among temperature treatments but only slight, non-significant shifts between the day and

night (generalized mixed models with day and chamber identity as random factors: $F = 15.8$, $p = 0.064$, $n = 319$ records).

Jars were checked 2-3 times a day to note developmental times and collect shed skins (exuviae) and dead individuals. The left hind femur of exuviae or dead nymphs was measured using the same stereomicroscope fitted with an ocular micrometer with 0.06 mm accuracy. Individuals from all populations and temperature treatments passed through four instars before moulting to adults, and sexual size dimorphism appeared at the third instar (sexual differences in hind femur length during the first and second instar: $F_{1,37} < 0.87$; $p > 0.35$; third instar: $F_{1,37} = 5.09$; $p = 0.030$; fourth instar: $F_{1,28} = 4.72$; $p = 0.038$; adult: $F_{1,23} = 22.1$; $p < 0.001$).

Experimental design and data analyses

We used a split-plot design with three factors: temperature, population and incubator chamber. Temperature and population were crossed, while chamber was nested in temperature (Fig. S1). The temperature factor had three levels: (1) 20º/10ºC day/night temperatures, (2) 25º/15ºC and (3) 30º/20ºC. Hereafter, we define these treatments as 20º, 25º and 30ºC for brevity. Within each temperature treatment, we assigned two incubators and four levels of the factor population (Vegarredonda, Ándara, Rasa and Traviesos). Five egg pods per population were allocated to each of two incubators within each temperature treatment, with the exception of the Vegarredonda population, in which we placed 2-3 egg pods per incubator due to a lower sample size (Fig. S1).

As response variables, we considered four early ontogenetic traits of the species, before sexual size dimorphism emerged. We took into account two morphological traits: Hind femur length at the first and second instar stage (hereafter HF_1 and HF_2).

HF_1 can be considered a proxy for egg size, since the size at birth depends mostly on genetic influence or maternal investment, while in older instars size may also depend on environmental conditions (Berner and Blanckenhorn 2006). In order to avoid pseudoreplication and to perform analyses at the clutch level, HF_1 and HF_2 averages per clutch were estimated. Then, we considered two traits associated with physiological rates: growth rate between the first and second instar, and duration of embryo development. Growth rate was calculated as $[Log_{10}(HF_2)- Log_{10}(HF_1)]/\Delta T$ where ΔT is the time from hatching to the first moult. As for hind femur lengths, individual values were calculated and then averaged to obtain means per clutch. The duration of embryo development after diapause, or timing of hatching, was calculated in days from the start of incubation to hatching. As hatching lasted up to 4 days per clutch, the average value per clutch was estimated.

We first performed factorial ANOVAs with a hierarchic design to ascertain the effect of temperature on the above traits, entering into the models temperature, chamber nested in temperature, population and two interactions (population × temperature, population × chamber nested in temperature) (Table S2). Since there was no chamber effect on traits (see Table S3), we proceeded with a similar design to test for the effect of elevation (low *vs*. high elevation), entering into the models elevation, population nested in elevation, temperature and two interactions (temperature × elevation, temperature × population nested in elevation) (Table S3). We ran two models per trait in order to assign the correct error mean square for the *F* test of differences among temperatures (i.e., the term chamber nested in temperature) and between elevations (the term population nested in elevation). Both the chamber in the first ANOVA, and the population in the second ANOVA, were random terms since they were nested, and they were not expected to differ a priori within each temperature

or elevation (Bennington and Thayne 1994). Pair-wise differences among the levels of each factor were analyzed by means of Fisher's LSD tests. We considered the positive covariation of genotypic (elevation or population) and environmental (temperature) effects as an evidence of cogradient variation, while a negative covariation was interpreted as countergradient variation (Conover and Schultz 1995). Covariation implied a significant effect of the factor temperature and of the factor elevation, with the elevation effect in the same (cogradient) or in opposite (countergradient) direction of the temperature effect. In order to analyse whether early development was subject to significant environment-genotypic interactions, we performed nested ANCOVAs in which body size at hatching and at the second instar were modelled on temperature, elevation, and time, with population nested in elevation. Time was quantified as the duration of embryo development for size at hatching, and the time to the first moult for size at the second instar. The size at the first instar was entered in the model for the second instar, to focus on development between the first and the second stage irrespective of the starting conditions. In the above analyses, the unit of the analysis was always the clutch, and analyses were performed on data standardized to zero mean and unit standard deviation.

We then quantified survival selection on duration of embryo development and body size at hatching by means of standardized selection gradients, in which data were analysed at the individual rather than at the clutch level as we centred on individual fitness. We considered each treatment separately since we aimed to measure phenotypic selection induced by low, medium and high temperatures. For this purpose, we standardized trait values to zero mean and unit standard deviation within each temperature treatment, and quantified relative fitness as nymph lifespan divided by the average lifespan of nymphs grown at the same temperature, with the maximum

lifespan value being the entire duration of the nymphal stage. We considered lifespan as a surrogate of viability, which is a major component of fitness (Benton and Grant 1999). By means of multiple regression of relative survival on individual trait values, we tested for the significance of linear (β coefficient) and quadratic (γ coefficient) selection gradients, which express the partial change in trait value (in units of standard deviation of the trait) due to the direct selection on them (Lande and Arnold 1983; Laiolo and Obeso 2012). When multiple traits are considered, the coefficients β and γ indicate selection directly on the trait of interest, controlling statistically for indirect selection due to correlated traits, which is especially important here because of the size - duration relationship (see below). As evidence for local adaptions, we investigated whether a significant gene-environment interaction conditioned fitness (Kawecki and Ebert 2004), and thus whether temperature differently affected high and low elevation nymphs. For this purpose, we ran mixed-effect ANOVAs and modelled relative fitness on the full cross between elevation and body size or duration of embryo development, with the population nested in elevation as a random term; the degrees of freedom were estimated by using the Satterthwaite method.

All statistical analyses were performed with R (R Development Core Team 2015).

Results

The dissection of egg pods after hatching revealed that approximately the same number of eggs was assigned to each treatment (20º and 30ºC: 248 eggs each, 25ºC: 246), and that clutches contained from 2 to 15 eggs with no significant differences among populations (average 7.0 ± 0.20 SE, $F_{3,102} = 1.33$, $p = 0.26$). We found a clear

differentiation among ontogenetic traits in their response to temperature and population, but non-significant effects of elevation (Table S2, S3). High temperatures accelerated growth rates ($F_{2,3} = 23.4$, $p = 0.011$) and shortened the duration of embryo development ($F_{2,3} = 274.3$, $p < 0.001$). In particular, growth rates were faster at 30ºC than at 25º and 20ºC (Fisher's LSD: all $p < 0.001$) while the duration of embryo development differed between all treatments (Fisher's LSD: all $p < 0.001$) (Fig. 2). Body sizes at hatching and at the second instar were not affected by either temperature (all $F_{2,3} < 3.44$, $p > 0.167$) or elevation (all $F_{1,2} < 1.70$, $p > 0.32$) but did vary among populations (HF_1: $F_{3,8} = 9.11$, $p = 0.011$; HF_2: $F_{3,7} = 5.23$, $p = 0.033$). In particular, nymphs of the lowland Vegarredonda population were larger than those of the other populations at the first (Fisher's LSD: all $p < 0.004$) and at the second stage (all $p < 0.012$) (Fig. 2). These differences mirrored size-variation in reared adults, in which Vegarredonda females and males were the largest-bodied individuals of our sample (Fisher's LSD: all $P < 0.001$) (Fig.3). There was a significant correlation of offspring size not only with parent female size, but also with parent male size (male hind femur vs. HF_1: $r = 0.9973$, $F_{1,2} = 372.3$, $p = 0.0026$; male hind femur vs. HF_2: $r = 0.9972$, $F_{1,2} = 356.2$, $p = 0.0027$),

Duration of embryo development also varied among populations ($F_{3,8} = 5.49$, $p = 0.024$) but not with respect to elevation ($F_{1,2} = 7.20$, $p = 0.11$). Significant pair-wise differences were found among the lowest elevation population, Vegarredonda, and the other populations at the warmest treatment (longer developmental times at 30º: Fisher's LSD: all $p < 0.029$) and also between Vegarredonda and the highest elevation population, Traviesos, at the intermediate temperature treatment ($p = 0.038$) (Fig. 2). Therefore, eggs from the warmest site had the slowest development at warm temperatures but temperature shortened hatching times, indicating opposing

population and temperature sources of variation. As an indicator of a slight influence of elevation on development, we found a significant interaction between the duration of embryo development, temperature and elevation in determining body size at hatching (ANCOVA: $F_{2,3}$= 17.11, P = 0.023). There seems to be a tendency for the largest sizes to be associated with long development times at low and medium temperatures, and in low elevation clutches, but the relationship is not marked (Fig. 4). The influence of this triple interaction, with moult time in place of hatching time, was not significant for body size at the second instar (ANCOVA: $F_{2,3}$ = 0.18, p = 0.84), which depended solely on body size at hatching ($F_{1,15}$ = 9.96; p < 0.01).

Selection favoured a larger size at hatching at all temperatures, although the relationship of relative survival with size was quadratic at 20ºC (30ºC: β = 0.20 ± 0.10 SE, t_{49} = 2.03; p = 0.0479; 25ºC: β = 0.26 ± 0.09, t_{86} = 2.93; p = 0.004; 20ºC: β = 0.09 ± 0.07, t_{62} = 1.21; p = 0.23; γ = 0.19 ± 0.06, t_{62} = 3.11; p = 0.003) (Fig. 5). At 25^{o}C, the slowest individuals at hatching also achieved high relative fitness, although when entering both duration and size in models the former effect was no more significant, as an indication that body size trades off with time at this temperature (duration vs. size: r_P = 0.33; t_{86} = 3.32; p = 0.013). At 20ºC, the significant quadratic effect of body size shows that small individuals also achieve a certain survival benefit, in combination with fast development that is also favoured at cold temperatures (β = -0.17 ± 0.08, t_{62} = 2.20; p = 0.03) (Fig. 5). The product of duration and size had no significant effect in any treatment, indicating no evidence of correlational selection on the two traits. Large-sized nymphs were favoured at all elevations since there was no significant interaction between elevation and body size in determining relative fitness. However, fast hatching resulted in increased fitness in highland individuals (mixed-effect ANOVA: elevation × duration: $F_{1,57.4}$ = 4.82, p = 0.032), although this effect is

due to one highland population (Rasa) being significantly faster than the others (population × duration: $t_{51} = 1.58$; $p = 0.041$). Finally, the partial regression coefficients of body size, growth rate and time to moult at the second instar were not significant at any temperature (all $t < 1.58$; $p > 0.12$), suggesting that selection on size and time only involved the previous stage.

Discussion

Growth opportunities change markedly across climate gradients, and the ability to schedule development in a way that optimizes fitness is a characteristic of ectotherms with complex life cycles (Altwegg and Reyer 2003; Skelly 2004; Alvarez et al. 2006). In the alpine *Chorthippus cazurroi,* the spatial proximity between populations at the lower and upper range boundaries likely hampered the differentiation of body size along elevation, which did no occur in either nymphs or the wild parental generation. However, we found selection for fast-developing embryos at cold temperatures, a strategy that was especially favourable to highland nymphs. In the following stage there was no evidence of compensation, body size depended exclusively on size at hatching, and growth rate on temperature. The intense post-hatching selection on growth and body size also disappears at this second stage.

Contrary to other Gomphocerinae from temperate zones (vanWingerden et al. 1991; Telfer and Hassall 1999; Berner et al. 2004), we found no direct effect of growing temperatures, or of the elevation of origin, on the size of nymphs, in spite of the fact that this varied among populations (Fig. 2). Gene flow may have prevented the full ecotypic differentiation along elevations in this species, since only the farthest populations significantly differed in size (Vegaredonda and Traviesos, separated by 900 m elevation and 3.8 km linear distance), but not the other extreme pair (Ándara

and Rasa, only 530 m elevation and 1.7 km distance). Indeed, most evidence on climate-induced variation in growth and development morphology in grasshoppers and other ectotherms has been obtained in well-isolated populations over broad latitudinal scales, where the opportunity for local genetic differentiation is great (Li et al. 1998; Hatle et al. 2002; Laugen et al. 2003; Parsons and Joern 2014). Moreover, local adaptations may be confounded by genetic drift when populations are small (Kawecki and Ebert 2004), which is the case of marginal lowland populations of *Chorthippus cazurroi*. In spite of this, the significant effect of population and non-significant effect of temperature on offspring size support the idea of a partial genetic differentiation among populations, or of strong maternal effects since this is a one-generation experiment. The idea of a genetic source of variation is partially supported by the significant correlation between nymph and adult male body size, but this has to be definitively tested over a larger number of generations.

In spite of the apparent lack of significant thermal and elevation influences on body size at hatching and at the successive stage, elevation and temperature did influence embryo development, and concurred in determining the future performance of nymphs. Being larger was better for survival in all populations and in all treatments (Fig. 5), a finding that is in line with results obtained by several studies on ectotherms, which also found selection on size to be strongly and significantly shifted to positive values of β (Kingsolver et al. 2001). In *Chorthippus cazurroi*, the strength of directional selection (β = 0.20 and 0.26) was within the range of values obtained in unmanipulated field populations, as reviewed by Kingsolver and Huey (2008) (literature median β = 0.15). The same occurred for selection on embryo development time (−0.17 against the median literature value of −0.145). Unlike to most field studies, we were able to include time together with size in models and thus investigate

a fitness correlation that has often been assumed, but that largely lacks empirical validation (Kingsolver and Huey 2008). We found that, at low temperatures, negative selection on development time opposed a positive direct selection on body size, curving the fitness relationship of body size (γ significantly differed from zero) (Fig. 5). However, β for body size was not significantly negative, as for disruptive selection favouring extreme sizes (Lande and Arnold 1983), thus there might be no direct fitness advantages of being small *per se*. Small sizes seem to be rather a by-product of selection for fast development, which is made possible by pleiotropy or linkage between genes codifying for ontogenetic traits, which bound hatching to the achievement of a critical threshold size (Roff 2002; Bernardo 1993; D'Amico et al. 2001; Berner and Blanckenhorn 2007). This fast strategy, widespread in ectotherms from cool climates, is the common outcome of countergradient selection stemming from time constraints, and permits small individuals to attain reproduction over a shorter growing and breeding season (Berven and Gill 1983; Dingle et al. 1990; Berner and Blanckenhorn, 2006). In our experiment, however, costs and benefits have to be estimated during the life stages observed here, thus individuals should be seen as maximizing their growth rate during these early stages. Results obtained at 20ºC, in which the fastest phenotypes were slow as compared to the warmest treatments, and the slowest ones represented the outmost fringes of trait value (Fig. 4), indeed suggest an optimization of development in this species.

In conclusion, our results do not support the idea of fully plastic phenotypes or cogradient variation, but indicate weak countergradient patterns of development, and size-time trade-offs underlying fitness during the early stages of life. Post-hatching selection was strong on embryo development and size at hatching, but disappeared in the successive stage, overall confirming that the onset phase of life is critical for the

future performance of individuals, as in many other animal groups studied in the wild (Verbeek et al. 2008; Kluen et al. 2011). Directional phenotypic selection on development times in embryos certifies the strength of selection imposed by cold temperatures in the laboratory, and highlights responses early in nymph life that are likely candidates for becoming local adaptations. In spite of this, elevation clines of body size did not occur in either nymphs or the wild parental generation, and no full countergradient covatiation between elevation and temperature was found in nymph development. Therefore, although divergent or directional natural selection stemming from climate may become a powerful evolutionary force, its action may be levelled out by fecundity selection (for large sizes; Honek 1993), by drift or gene flow, or by micro-climatic differences within sites that reduce the likelihood of directional selective pressures (Sømme 1989), features associated with alpine environments or ectotherm life styles.

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Ethical standards

Experiments comply with the current laws of Spain. Grasshoppers were captured with permission of the Picos de Europa National Park (N. Ref. CO/08/058/2012).

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Graphic representation of possible scenarios of phenotypic variation in response to a new environment (transplant or common garden, either low L or high H environments in the x-axis), modified after Conover and Schultz (1995), and Ghalambor et al. (2007). Phenotypic values in the native site are represented by filled circles, here defined as two ecotypes. Arrows represent the phenotype that a genotype would express when introduced into the novel environment. Panel a – Two ecotypes display no phenotypic plasticity. Panel b – Phenotypic differences between sites are exclusively due to plasticity. Panel c – Two ecotypes have different degrees of plasticity (i.e., different slope of the reaction norm). Panel d – Two ecotypes have the same degree of plasticity (similar slope of the reaction norm), but have divergent phenotypes when each is measured in their native habitat. When measured in a common environment, they are still different, but the plastic response reduces the difference between them. Panel e –Two ecotypes have the same degree of plasticity, and when measured in their native habitat, they have a similar phenotype. However, when measured in a common garden, the plastic response increases divergence.

Fig.2 Variation in body size (hind femur length) at the first and second instar, in growth rate and duration of embryo development among four populations of *Chorthippus cazzuroi* in response to temperature variation. Raw mean values ± SE are shown.

Fig. 3 Body sizes of adult females and males of the parental generation. Mean values ± SE of hind femur length are shown.

Fig. 4 Relationship between body size at hatching and duration of embryo development at different temperature treatments and elevations. Data were standardized to zero mean and unit standard deviation.

Fig. 5 Regression of nymph relative fitness, quantified by relative lifespan, on the duration of embryo development or body size at hatching at the three temperature treatments. Each point represents an individual within the treatment, and the slope of the fitted line (with grey-shaded CI) is the selection gradient. Since we estimated selection gradients on two traits, size and duration, lines show partial effects, removing the effect of the other trait when significant. Trait values were standardized within each treatment; only significant trends are shown.

Supplementary material

Table S1 Topographic and climatic characteristics of the study populations.

Fig. S1 Graphical representation of the experimental design, including three factors: Temperature (three levels), Population (four levels) and Chamber (two levels within temperature treatments). Temperature and Population were crossed and Chamber was nested within Temperature.

Table S2 Results of factorial ANOVAs aimed at testing the influence of temperature on hind femur length at the first instar, hind femur length at the second instar, growth rate, duration of embryo development.

Table S3 Results of factorial ANOVAs aimed at testing the influence of elevation on hind femur length at the first instar, hind femur length at the second instar, growth rate, duration of embryo development.

Fig. 1

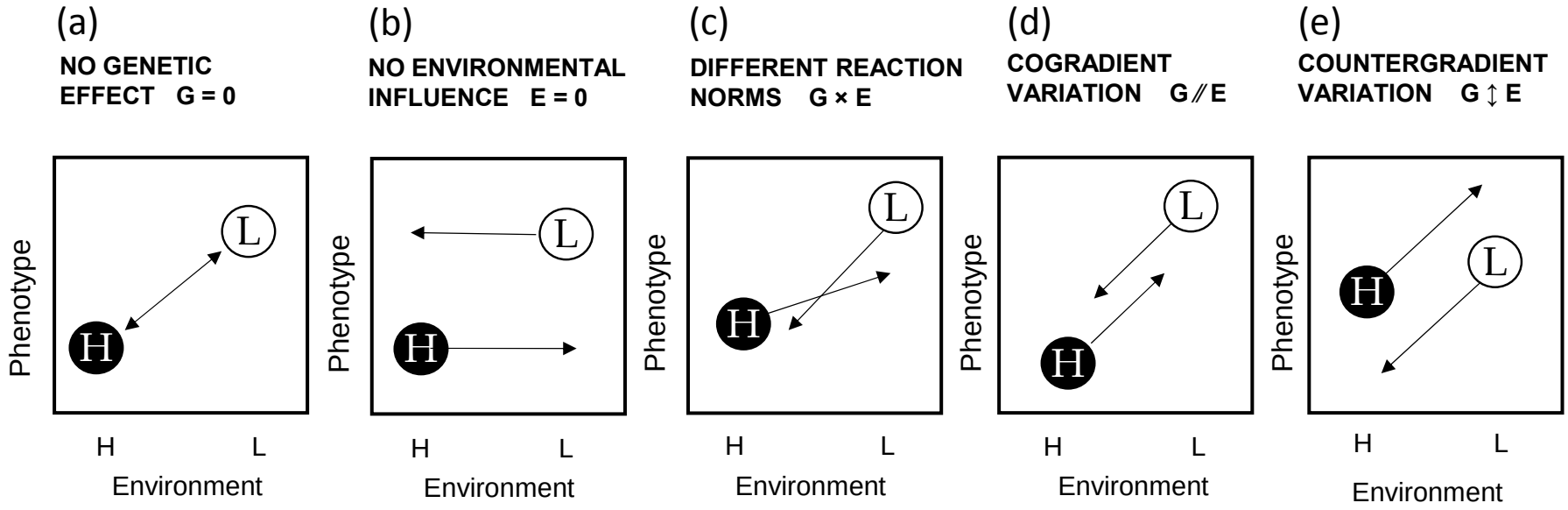

(a)
NO GENETIC EFFECT G = 0
(b)
NO ENVIRONMENTAL INFLUENCE E = 0
(c)
DIFFERENT REACTION NORMS G × E
(d)
COGRADIENT VARIATION G∥E
(e)
COUNTERGRADIENT VARIATION G↕E
Phenotype
H
L
Environment

Fig. 2

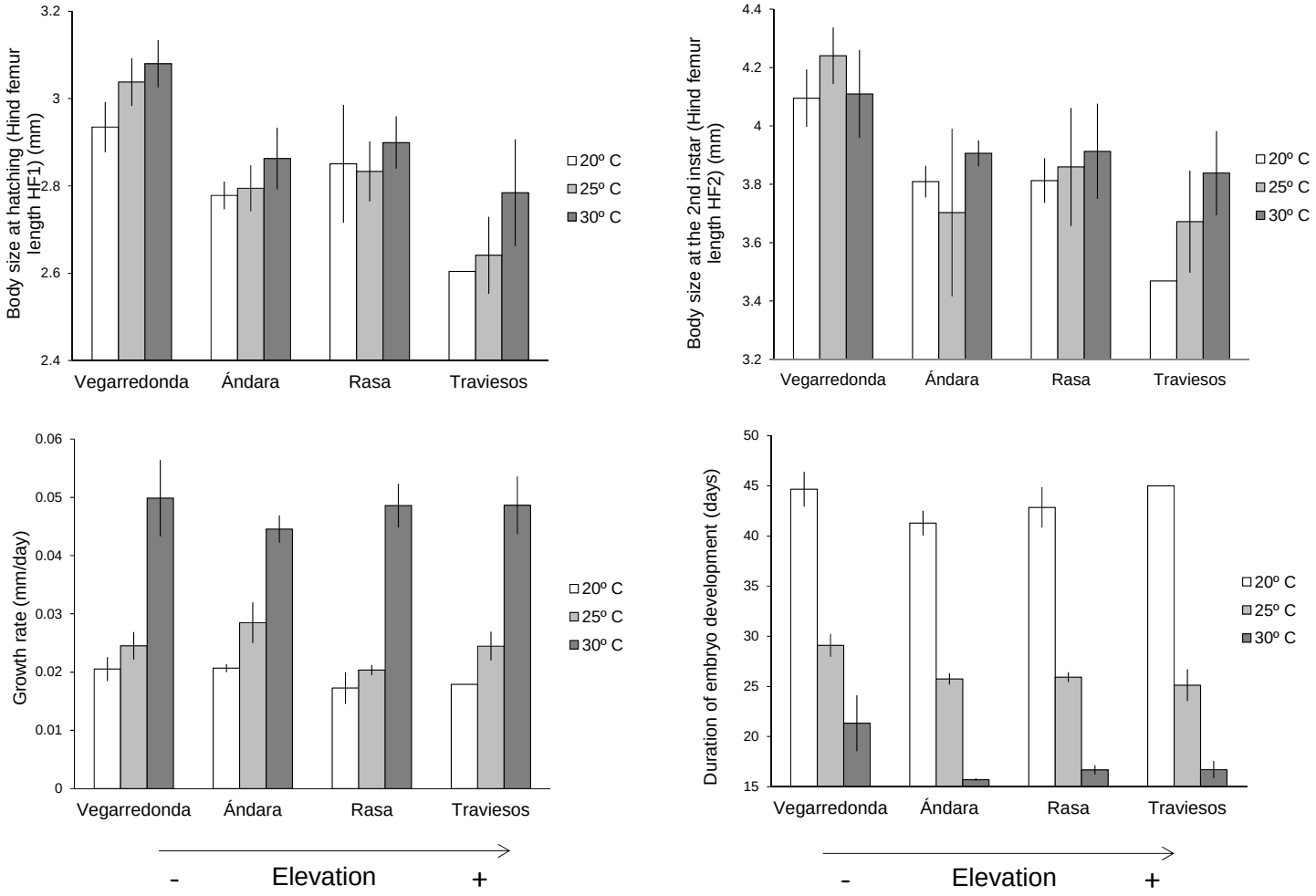

Body size at hatching (Hind femur length HF1) (mm)
3.2
3
2.8
2.6
2.4
Vegarredonda
Ándara
Rasa
Traviesos
20º C
25º C
30º C
Body size at the 2nd instar (Hind femur length HF2) (mm)
4.4
4.2
4
3.8
3.6
3.4
3.2
Growth rate (mm/day)
0.06
0.05
0.04
0.03
0.02
0.01
0
Duration of embryo development (days)
50
45
40
35
30
25
20
15
- Elevation +

Fig. 3.

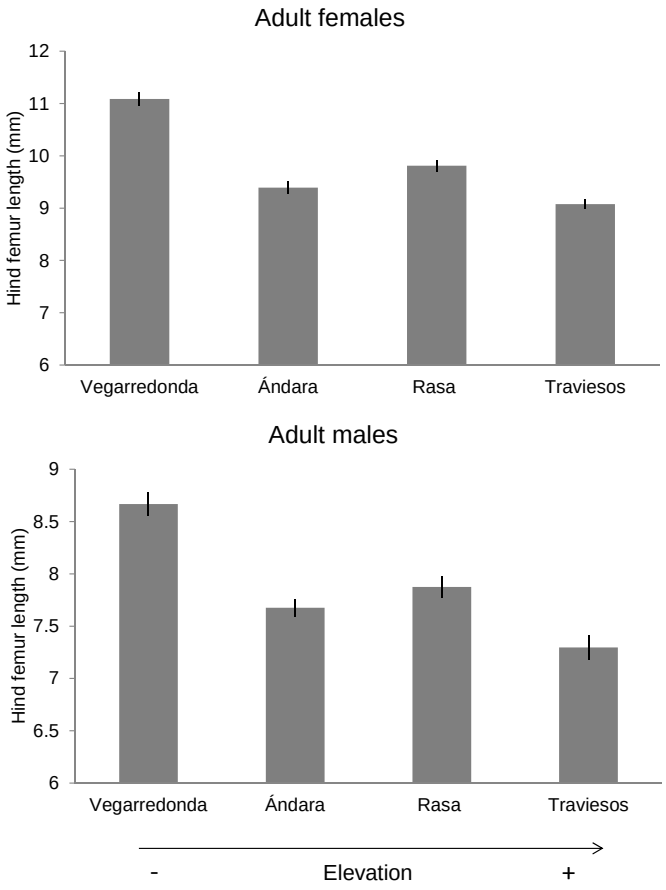

Adult females
Hind femur length (mm)
12
11
10
9
8
7
6
Vegarredonda
Ándara
Rasa
Traviesos
Adult males
Hind femur length (mm)
9
8.5
8
7.5
7
6.5
6
Vegarredonda
Ándara
Rasa
Traviesos
-
Elevation
+

Fig. 4.

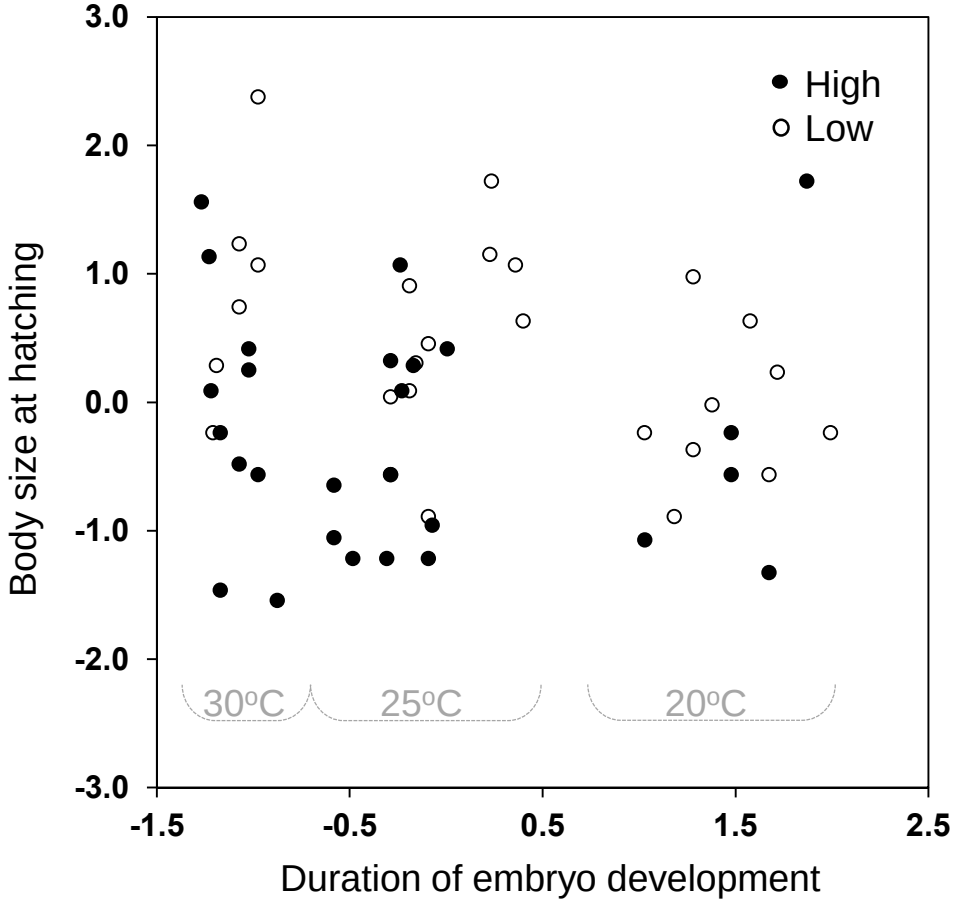
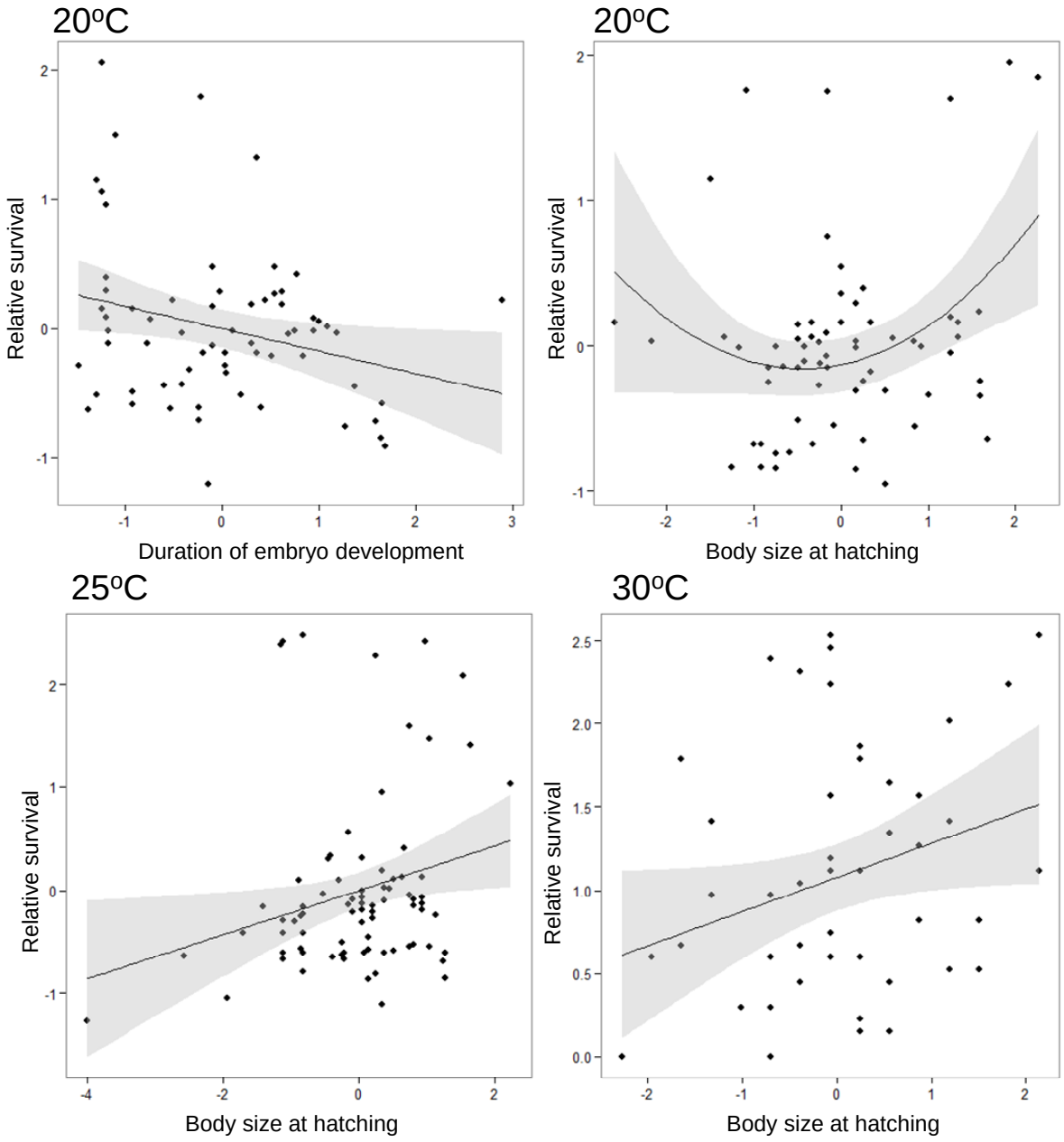

3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-1.5
-0.5
0.5
1.5
2.5
Body size at hatching
Duration of embryo development
High
Low
30°C
25°C
20°C

Fig.5.


20ºC
Relative survival
Duration of embryo development
20ºC
Relative survival
Body size at hatching
25ºC
Relative survival
Body size at hatching
30ºC
Relative survival
Body size at hatching