

Selection for functional performance in the evolution of cuticle hardening mechanisms in insects

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Calcified tissues have repeatedly evolved in many animal lineages and show a tremendous diversity of forms and functions. The cuticle of many insects is enriched with elements other than Calcium, a strategy of hardening that is taxonomically widespread but apparently poorly variable among clades. Here, we investigate the evolutionary potential of the enrichment with metals in insect cuticle at different biological levels. We combined experimental evidence of Zinc content variation in the mandibles of a target species (*Chorthippus cazurroi* [Bolívar]) with phylogenetic comparative analyses among grasshopper species. We found that mandibular Zinc content was repeatable among related individuals and was associated with an indicator of fitness, so there was potential for adaptive variation. Among species, Zinc enrichment evolved as a consequence of environmental and dietary influences on the physical function of the jaw (cutting and chewing), suggesting a role of natural selection in environmental fit. However, there were also important within and transgenerational environmental sources of similarity among individuals. These environmental influences, along with the tight relationship with biomechanics, may limit the potential for diversification of this hardening mechanism. This work provides novel insights into the diversification of biological structures and the link between evolutionary capacity and intra- and interspecific variation.

KEY WORDS: Biomechanics, evolvability, grasshoppers, plant defenses, sexual dimorphism, transition metals.

The calcified tissues of animals, such as shells, bones, cartilage, and enamel, record a broad range of information over physiological, ontogenetic, and evolutionary time scales and display a tremendous diversity of forms and functions (Carlson 1990; Vermeij 1995). Their diversification affects all levels of biological integration and is associated with high degrees of evolvability, that is, the ability to channel genetic variation into adaptive phenotypic variation and to differentiate in response to changing environmental conditions in the course of evolution (Jernvall 2000; Knoll 2003). Calcium is not, however, the only element that animals incorporate to achieve resistance to pressure and deformation. Many insect taxa accumulate Zinc, Iron, and Manganese in their cuticle (Hillerton and Vincent 1982; Cribb et al. 2008a,b). The incorporation of these transition metals has broad ecological (Buchwalter and Luoma 2005), agronomical (Hillerton et al. 1984), and nutritional (Mwangi et al. 2018) im-

plications, also outside the entomological domain, but has been poorly studied from a functional and evolutionary perspective. It has been linked to trophic specialization, but a variety of herbivorous, xylophagous, granivorous, and carnivorous taxa have metal-enriched mouthparts (Hillerton and Vincent 1982; Quicke et al. 1998; Morgan et al. 2003; Cribb et al. 2008b; Polidori et al. 2020). It also appears to have an ancestral origin and resists to changes along species evolutionary history (Polidori et al. 2020). Yet, no quantitative study has addressed the critical aspects that define adaptive and evolutionary potential, that is, has tested whether variation among individuals is functional and heritable, and whether ecological diversification promotes variation among species (Losos 1994).

Here, we addressed the development, function, and evolution of the location and extent of mandible metal enrichment in grasshoppers (Orthoptera, Acrididae) to link micro- and

macroevolutionary aspects that define the evolvability of this character. Previous studies on grasshoppers have found enrichment with Zinc that increases cuticle fracture toughness (Hillerton and Vincent 1982). Moreover, grasshopper mandible morphology and strength vary with the composition and mechanical properties of plant food (Patterson 1984; Ibañez et al. 2013a,b). The link between cuticle hardening, mandible biomechanics, and trophic niche is, however, missing. Grasshoppers consume food in proportion to their weight, being females heavier than males (Hewitt 1977; Laiolo et al. 2013), but the consequences of sexual dimorphism for cuticle hardening are unknown. In this study, we first addressed variation in this trait among relatives and individuals within species. We tracked changes from hatching to adulthood in a specialist graminivorous grasshopper in a common garden experiment. Through full-sib and mother-offspring correlations, we assessed the potential for inheritance and environmental factors (mother and offspring age) as sources of variation. We then analyzed the relationship of Zinc content with cutting and chewing power (Ibañez et al. 2013a), to assess its functional significance, and with offspring body size, a crucial life history trait that is simultaneously a maternal and offspring fitness correlate (Mousseau and Dingle 1991; Sinervo et al. 1992). At the macroevolutionary scale, we measured the statistical dependence among the aggregated Zinc enrichments of grasshopper species due to their phylogenetic relationships (phylogenetic signal). We then tested its environmental fit (to climate, habitat, diet) and the influence of intrinsic constraints among species (sexual dimorphism, allometric relationships). If Zinc content has a high evolutionary potential, then variation in this trait should correlate with fitness, be inheritable, adjust to environmental and dietary shifts, and be weakly constrained by intrinsic biological factors such as body size and phylogeny. On the contrary, a significant influence of environmental conditions during development and parental effects, and strong intrinsic constraints to variation at the macroevolutionary scale, would indicate that the evolutionary potential and capacity for differentiation is reduced.

Material and Methods

STUDY SYSTEM

Development and fitness correlates of mandible Zinc content were studied in a lab-reared first generation of *Chorthippus cazurroi* (Bolívar 1898) individuals. This species is univoltine, grass-feeder, and endemic to the Cantabrian Mountains (NW Spain) (Laiolo and Obeso 2015; Illera et al. 2019). Grasshoppers of the parental generation were collected alive in the Picos de Europa National Park early in summer 2013. In the laboratory, adult individuals were housed in pairs in individual cages, whereas individuals collected as nymphs, at the first instar stage,

were initially raised together and then paired in individual cages after molt to adults. The cage floor was filled with sand to allow females to lay, and egg-pods were collected and stored daily. After egg diapause and hatching (details in Pato et al. 2019), in spring 2014, we raised the first generation of nymphs from the above pairs, placing in a common cage those sibs from the same pod that emerged in the same day. Cages were checked daily to remove dead individuals and exuviae, which were frozen for further analyses. Four molts and about 40 days after hatching, adults emerged. One adult sib per pod and per sex was frozen at emergence and the rest were raised with a nonrelative member of the other sex until natural death, to allow reproduction. These individuals also were frozen for further analyses. Throughout the duration of the experiment, individuals were exposed to natural light plus light bulbs as heat sources during the day (26–33°C day/19–23°C night). They were fed ad libitum on a fresh mixture of relatively hard monocots typical of the diet of the species (*Brachypodium pinnatum*, *Bromus erectus*, *Dactylis glomerata*, and *Carex brevicollis*). In addition, we also provided wheat leaves to nymphs.

Among successful pairs, 22 were formed by mates that were collected as nymphs, developed in the laboratory, and formed full-sib families. These families, with two generations reared almost entirely in the lab, were used for studying transgenerational genetic effects (repeatability analysis, see below). Another 17 pairs included females collected as adults that developed, and might have mated, in the field, thus their offspring may include half-sibs. These latter individuals were used together with full-sibs to analyze intragenerational relationships (function and development, see below).

The evolutionary change of Zinc content was studied in 29 Acrididae species inhabiting the Cantabrian Mountains. We collected over 8000 individuals belonging to the subfamilies Oedipodinae, Melanoplinae, Calliptaminae, and Gomphocerinae, at 189 sites in the period 2012–2018. The survey area covered 20,000 km² and the entire elevational gradient of the Cantabrian Mountains, from sea level to mountain tops at over 2600 m above sea level (Laiolo et al. 2015, 2018, 2020).

MANDIBLE MEASUREMENTS

We focused on the left mandible for this study, in keeping with Patterson (1984) and Ibañez et al. (2013a,b). The mandibles dissected from dead individuals or exuviae were dried and gold coated for the scanning electron microscope study. Electron images were recorded on a JEOL JSM-6610 LV operating at 20 kV, with a working distance of 10 mm and a Spot Size of 42 μm. The JEOL JSM-6610 LV was fitted with an EDX Inca energy-350 X-ray image analyzer and an X-Max 50 X-ray detector. The spectra were taken with a live acquisition time of 60 s. Data were acquired and processed with the INCA Suite version 4.15 and the

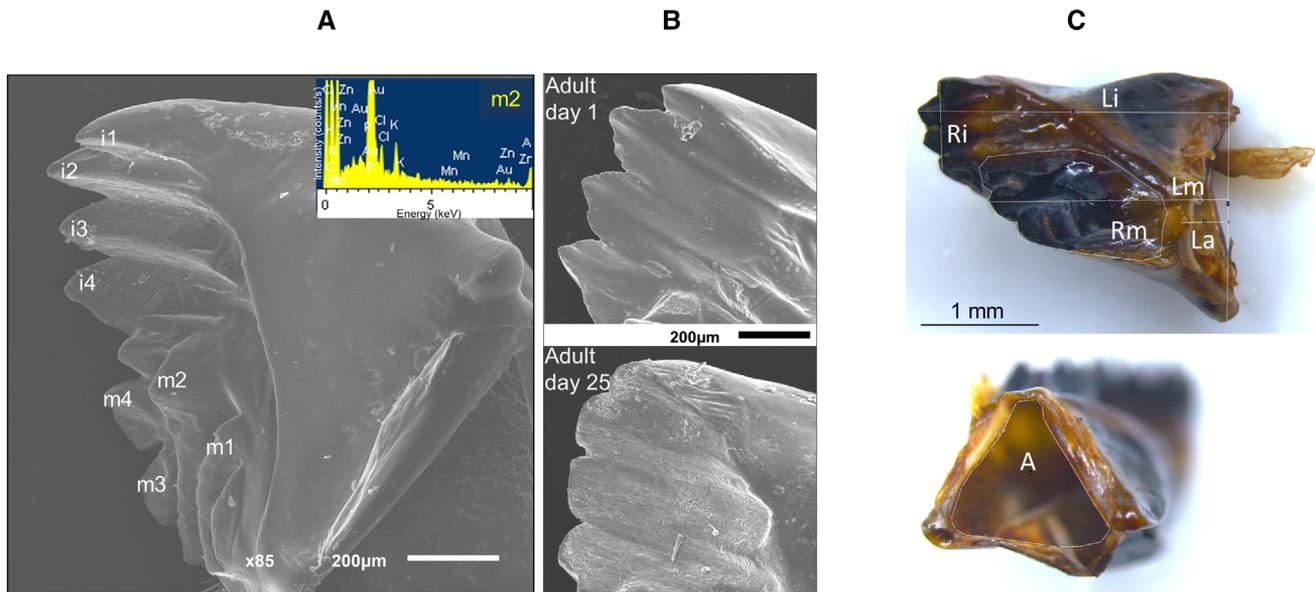


Figure 1. Grasshopper mandible images. (A) Scanning electron micrograph of the left mandible of *Chorthippus cazurroi*, with the locations of Zinc quantification (incisive ridges from i1 to i4, molar ridges from m1 to m4) and an X-ray microanalysis spectrum of location m2. (B) Scanning electron micrographs of the incisive ridges of two *Chorthippus cazurroi* adults of different ages, in which teeth wear can be appreciated in the older individual (bottom). Pictures by Joaquina Pato. (C) Picture of the left mandible of *Psophus stridulus*, at different angles, with the segments and surfaces measured for the biomechanical study (Li: incisive lever; Ri: incisive length; Lm: molar lever; Rm: molar area; La: adductor muscle lever; A: mandible section area). Pictures by Marta Alonso.

reference spectrum for elements was calibrated using Mn and Cu standards. The concentration of each element was determined after applying matrix corrections with the $\Phi(\rho z)$, XPP, approach to define the shape of the spectrum curves, as described in Polidori et al. (2013). Zinc was the most abundant metal in the mandible and was located at the edges of incisors and molars (Figs. 1 and S1; Table S1). Its abundance was determined using the peak to background abundance, and expressed as percent content (mass % dry weight) rounded to the nearest 0.05%, because noise variance (% weight σ^2) exceeded one-half of the measurement for concentrations lower than 0.05% (Duncumb 1994). To further reduce measurement bias, we averaged the concentrations in four incisor tips to obtain Zinc percent abundance in the incisive ridge, and the concentrations in four molar tips to calculate Zinc percent abundance in the molar area (Fig. 1A). In incisors, none of the interspecific sample and 4% of the intraspecific one presented low concentration (<0.3 weight %; Polidori et al. 2013), whereas in molars regions low concentrations were found in 13% of inter- and 31% of intraspecific specimens. We took measurements in 91 adult individuals of the first generation (49 from full-sib and 42 from half-sib families) and 22 females of the parental generation. Furthermore, to track changes throughout the life cycle, from hatching to maturity, we measured mandibular Zinc content in 10 unrelated females of the first generation sacrificed at emergence and in their first, second, third, and fourth exuviae (40 exoskeletons). For each of these females, we also measured the Zinc

content of one of its sibs that died a few days after hatching (thus at the first instar stage), and of one sister that completed the life cycle up to reproduction (average age after molt: 25 days). The mandibular Zinc of 11 additional first-stage nymphs was measured to analyze shifts at the very beginning of grasshopper life (from hatching to day 6). In the Acrididae community, we measured Zinc content in the mandibles of one male and one female of 29 species. Only specimens with mandibles that were fully pigmented and sclerotized, indicative that all were mature adults, were considered.

To assess the functional significance of mandible Zinc content, we analyzed its relationship to the strength of the mandible. This was estimated using the biometric-biomechanical model developed for Orthoptera by Ibañez et al. (2013a). We took calibrated multifocus pictures of mandibles with a Leica M205FA stereomicroscope and a Leica DFC310FX color digital camera (Leica Microsystems GmbH, Mannheim) (Fig. 1C). Images were acquired with LAS V4.0 (Leica Application Suite, Version 4.0.0) and measurements were taken by ConfocalUniovi ImageJ 1.51 (<http://spi03.sct.uniovi.es/confocaluniovi/confocaluniovi.htm>), a bundle of ImageJ (NIH ImageJ 1.49p, <https://imagej.nih.gov/ij/>) developed by Angel Nistal at the Photonics Microscopy and Image Processing Unit of Oviedo University (Spain). With these tools, a set of mandible portions was measured to obtain proxies of incisive strength ($FI = A La/Li$ 1/Ri) and molar strength ($FM = A La/Lm$ 1/Rm) (Fig. 1C) following the model developed

by Ibañez et al. (2013a). In this model, La, Lm, and Li represent the lever arms, Rm and Ri the stress area or segment (where the force is applied), and mandible section area A approximates the adductor muscle force (Ibañez et al. 2013a). Mandible strengths were estimated in 71 adults of *Chorthippus cazurroi* and in two to eight individuals per sex and species of the Acrididae community.

BIOLOGICAL CONSTRAINTS AND ENVIRONMENTAL SELECTIVE AGENTS

We derived a set of phylogenetic, ecological, and environmental predictors for interspecific comparative analyses. We reconstructed the phylogeny of the study community using a 578 base-pair fragment of the mitochondrial DNA Cytochrome Oxidase subunit I obtained from the sample we collected in the field (Table S2; Fig. S2). By means of a Leica stereomicroscope, we measured hind femur length, an indicator of body size that can also be measured in exuviae (Laiolo and Obeso 2015). The length of the femur correlates with another frequently used measure of body size in insects, total body length, both within *Chorthippus cazurroi* (Laiolo et al. 2013) and among species (this study: $r > 0.93$, $t_{27} > 14.1$, $P < 0.001$ in the two sexes). To establish male and female diet, food items were categorized into two main types that are known to contribute to grasshopper mandible shape: the relative proportions of dicots (forbs) and monocots (sedges and grasses) (Patterson 1984). We solely considered monocots proportion in analyses given that the percentage of dicots equals 1 minus the proportion of monocots in our sample of herbivorous grasshoppers (Table S3). We used information obtained from our own laboratory assays and molecular procedures, as well as from literature, because diet categories (monocots and dicots) are broad enough to encompass regional differences in plant species composition. In a sample of nine species in which both own and literature data were available, our observations and those of other sources were in fact highly correlated ($r = 0.90$, $t_7 = 6.1$, $P < 0.001$). Two climatic and habitat features were considered as environmental agents of selection (Laiolo et al. 2013). The average relative cover of grasslands in presence plots was used as a proxy of the availability of edible plants of species, which may affect trophic choices (Chown and Gaston 2010). The average annual temperature of plots, obtained from the digital layers of the Climatic Atlas of the Iberian Peninsula (Ninyerola et al. 2005), displays ample variation across the 2600 m elevation gradient of this study and may affect grasshopper development, body size, and diet (Laiolo et al. 2013; Laiolo and Obeso 2015). We assume that these features describe the environmental conditions in which grasshopper mandibles evolved, as deterministic processes influence species distribution (environmental filtering) and phenotypic traits (environmental fit) (Laiolo et al. 2018, 2020).

DATA ANALYSIS

Within and transgenerational variation

We analyzed the development of relative Zinc content in incisors and molars throughout the life cycle of *Chorthippus cazurroi* in the longitudinal sample of 10 females measured in exuviae at stage 1, 2, 3, and 4 and in sisters at emergence and hatching, by means of paired *t*-tests. The same statistics was used to estimate sexual differences in Zinc content between adult full sibs, whereas linear models served to analyze changes from hatching to the first molt in the cross-sectional sample of nymphs at stage 1. We then estimated the repeatability of molar and incisive Zinc content and its 95% confidence intervals within 22 full-sib families, both through full-sib correlations and mother-offspring correlations. With the R package *heritability* (Kruijer et al. 2015), we estimated repeatability as the intraclass (family) correlation coefficients among full-sibs as $V_g / (V_g + V_e)$, where $V_g = (\text{Mean Square [Family identity]} - \text{Mean Square [Error]})$ and $V_e = \text{Mean Square [Error]}$. In these analyses, we excluded the variance attributable to covariates significantly affecting Zinc content, as identified by my means of type-III ANOVAs, among the following predictors: age (number of days after the last molt), body size (femur length), and brood effects (egg-pod order). These repeatability values represent an upper limit to broad-sense heritability, because family effect includes all types of genetic contribution plus potential parental environmental effects (Kruijer et al. 2015). To explore the latter in greater depth, we analyzed the effect of mother's age on mother-offspring correlations in Zinc content. For this, we used factorial regressions in which we also controlled for offspring age.

Function and fitness correlates

To test whether mandible Zinc content was significantly associated with the stress applied in each region, we performed generalized linear mixed models with the R package *lmerTest* (Kuznetsova et al. 2017). Family identity was entered as random factor, and age as covariate. We then analyzed the fitness correlates of Zinc enrichment, testing for the association of the Zinc content in mothers' incisors (or molars) to the body size of daughters, measured at hatching in the exuviae and then at adulthood after emergence. Both body size values are indicators of grasshopper fitness: larger nymphs have higher survival (Laiolo and Obeso 2015) and larger adult females have higher fecundity (Branson 2008; Davidowitz 2008). We performed linear models in which we controlled for mother's body size and mother's molar (or incisor) Zinc content.

For residuals to be normally distributed, Zinc content values of *Chorthippus cazurroi* were transformed to Log+1 prior to fitting models. All *P*-values refer to two-tailed tests.

Evolution and coevolution among sexes

We first estimated the amount of phylogenetic signal in the Zinc content of species by means of two indices, Pagel's (1999) lambda and Blomberg et al.'s (2003) K , with the *phylosig* function of R package *phytools* (Revell 2012). Pagel's lambda measures the similarity of trait covariances among species to trait covariances expected under a (neutral) Brownian motion (BM) model of evolution, and Blomberg's K measures the partitioning of variance within and among clades with respect to a BM reference.

We then calculated average values per Acrididae species and sex of incisive and molar strengths and femur length. Average values per species were also calculated for grass cover and average temperature, and female and male diets were expressed as the relative percentages of monocots. These variables served to analyze the relationships between mandible Zinc variation among species and biomechanical traits (incisive and molar strengths), ecological niche correlates (monocots in diet and body size), and environmental factors (temperature and grass cover). Preliminary tests were conducted to assess whether the evolutionary trajectories of these traits were coordinated or independent, and whether there was an influence of phylogeny on the patterns of covariation. We tested associations between single variables and among groups of variables using univariate and multivariate least squares methods, respectively. We performed univariate phylogenetic generalized least squares (GLS) models between pairs of variables with the *ppls* function of the *caper* R package (Orme et al. 2018). Then, we analyzed the association between biologically meaningful groups of variables, such as ecological niche variables (2 variables), biomechanical variables (2), or larger blocks, as mandible variables (4), and so on. Without accounting for species phylogeny, we run two-block partial least squares analyses with the *two.b.pls* function of the *geomorph* R package (Adams et al. 2017). Taking into account phylogeny, we fitted multivariate GLS with the *mvpls* and *manova.gls* functions of *mvMORPH* R package (Clavel et al. 2019). Finally, we analyzed multiple relationships between Zinc content versus the rest of variables (considered separately: diet, size, incisors and molar strengths, temperature, and grass cover). These multiple models were run separately for each mandible region (phylogenetic GLS regressions) or jointly (multivariate GLS models accounting for phylogeny). The above exploratory analyses served us to identify significant relationships and build a set of biologically plausible multivariate models on the evolutionary diversification of Zinc content across species. We used piecewise structural equation modeling (SEM) (Shipley 2009; Lefcheck 2016) and the *psem* function of the R package *piecewiseSEM* (Lefcheck 2016) to string together individual GLS models (causal models) and simple correlations among the differ-

ent variables mentioned above. GLS models were run with the function *gls* of the R package *nlme* (Pinheiro et al. 2020) considering both phylogenetically independent scenarios and phylogenetic structures derived from BM (*corBrownian*) or Pagel's lambda (*corPagel*) models of trait evolution, with the *ape* R package (Paradis and Schliep 2019). The resulting multivariate models included different numbers of correlations and causal relationships among variables, but environmental variables were always hypothesized to be (causal) predictors (see also Results). Fisher's C test (Lefcheck 2016) was performed to assess model reliability and those models separated by less than 2 AIC points from the model with the lowest AIC were considered as equally probable.

Finally, we assessed whether female and male Zinc content changed in concert across species, given the large differences in sexual size dimorphism in the Acrididae (Laiolo et al. 2013). For this purpose, male Zinc content values were regressed on female values in interaction with body size dimorphism (female/male femur length) by means of phylogenetic GLS models fitted with the *ppls* function as detailed above. Given the significance of this interaction in incisors (see results), all the above comparative analyses were run separately in the two sexes.

Results

WITHIN AND TRANSGENERATIONAL VARIATION

Chorthippus cazurroi nymphs at hatching had a proportionally high relative content of Zinc in their mandibular ridges ($\approx 2\%$ in incisors and 1% in molars) (Fig. 2A). At each molt some of this element was lost, and the mandibles of freshly emerged adults were especially Zinc poor (Fig. 2A). However, the Zinc content in mature adults did not differ from that of hatchlings, despite body size (femur length) increasing by almost four times (Fig. 2A) (Table S4). It neither differed between full-sib adult male and female of comparable age (paired t -tests, $t_5 < 1.6$, $P > 0.16$) despite the marked sexual size dimorphism ($t_5 = 13.5$, $P < 0.001$). During the first nymphal stage, Zinc content did not increase over time (all $t_9 > 0.75$, $P > 0.47$) (Fig. 2B). The repeatability of mandibular Zinc content among adult full-sibs was significantly different from zero (incisors: 0.32, 95% CI: 0.03–0.64, molars: 0.39, 0.09–0.69, $n = 49$ individuals) after controlling for significant covariates (highlighted in Table S5). The estimated repeatability of molar Zinc content further increased when calculated separately among recently molted (0.78, range: 0.30–0.94, $n = 20$) and mature (0.61, range: 0.04–0.85, $n = 27$) adult sibs, given that age significantly affected concentrations in this mandibular region (minimum in recently molted individuals; Table S5). Mother-daughter correlations were significant in the case of

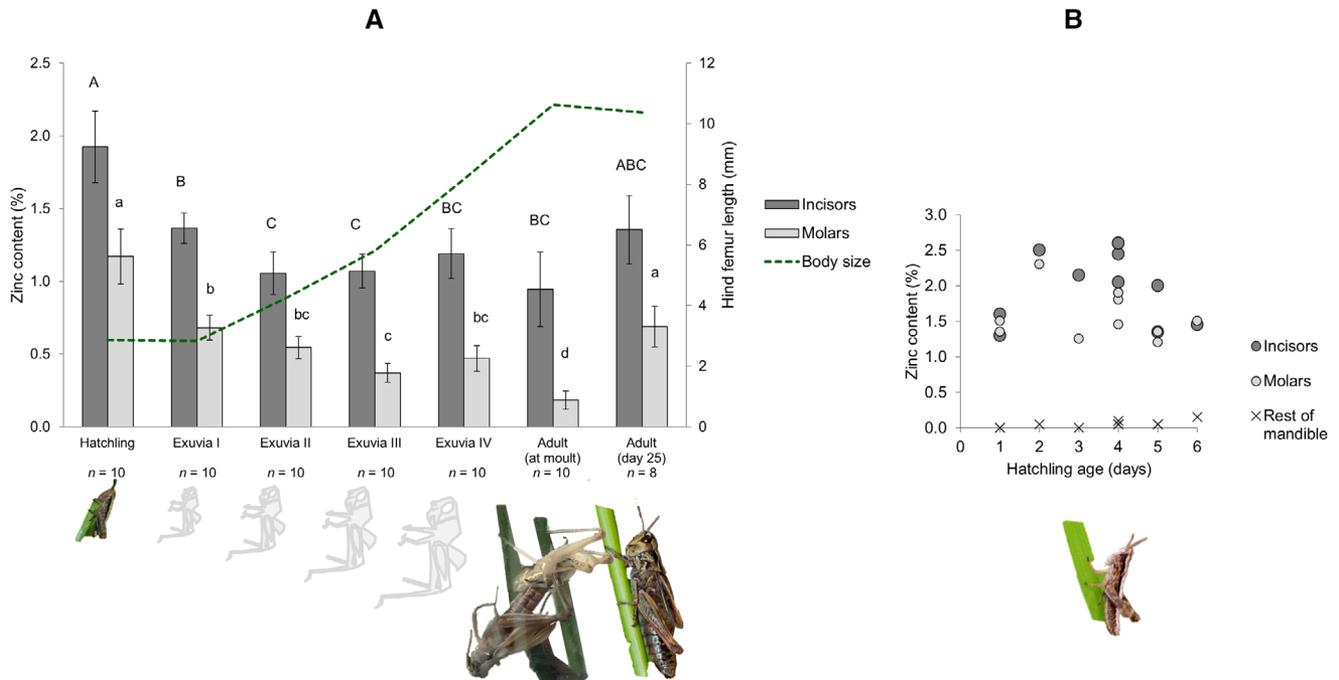


Figure 2. Ontogenetic changes of mandibular Zinc content in *Chorthippus cazurroi*. (A) Relative percentages of Zinc content in incisors and molars during the entire life cycle. Bars represent average values in a longitudinal sample of 10 females recorded from the first molt to the adult at emergence, and differences with their sibs at hatching and at maturity (average age 25 days). Changes in body size are represented by a green line. Differences among stages were tested by means of paired t-tests (see Table S4), upper cases refer to differences (at $P \leq 0.05$) in the incisor region, and lower cases to differences in the molar region, and similar letters indicate similar values across stages. (B) Relative percentages of Zinc content in incisors, molars, and in mandible center from hatching to the first molt in a cross-sectional sample of 11 grasshoppers at the first instar stage. Pictures by Paola Laiolo and Eva de Mas.

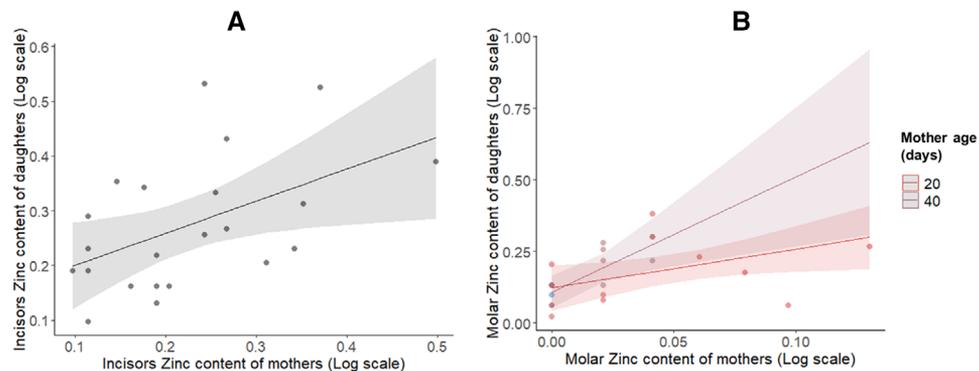


Figure 3. Mother-daughter relationships in Zinc content of incisors (A) and molars (B) of *Chorthippus cazurroi*. The regression line and 95% confidence intervals after controlling for the effects of daughter and mother age are shown. In the case of molar Zinc content (right panel), the relationship was affected by a significant interaction with mother age. Therefore, for representative purposes only, the predicted trends are shown for mothers of two different age classes (mature and old females).

incisors Zinc content (Estimate = 0.60 ± 0.25 SE, $t_{20} = 2.38$, $P = 0.029$) with no effect of mother and offspring age (all $P > 0.67$) (Fig. 3A). They were also significant in the case of molars after controlling for within and intergenerational age effects, and accounting for the interaction with mother age (Estimate = 0.13 ± 0.06 SE, $t_{16} = 2.15$, $P = 0.046$) (Fig. 3).

FUNCTIONAL AND FITNESS CORRELATES

In the mature adults of both sexes, the Zinc content of a given mandible portion was significantly and positively associated with the stress applied there (Table 1). The significant interaction with age in Table 1 reflects the lack of fit of this relationship in recently molted adults. These individuals still have an unworn mandible,

Table 1. Effects of individual sex and age on the relationships between mandibular Zinc content in a given mandible portion and the strength applied to that portion in *Chorthippus cazurroi* adults. Results of linear mixed effect models are shown, in which the Satterthwaite approximation was used to estimate the effective degrees of freedom. Zinc content values were log-transformed prior to analyses.

| Incisor Zinc content | Estimate | SE | <i>df.</i> | <i>t</i> -value | <i>P</i> -value |
|-------------------------------|-----------------|-----------|-------------------|-----------------------|-----------------------|
| Intercept | 0.466 | 0.092 | 57.905 | 5.094 | <0.001 |
| Sex | 0.037 | 0.217 | 62.846 | 0.170 | 0.866 |
| Age | -0.007 | 0.005 | 55.834 | 1.548 | 0.127 |
| Incisive strength | -0.628 | 0.333 | 53.423 | 1.886 | 0.065 |
| Sex × Age | -0.006 | 0.01 | 58.715 | 0.628 | 0.532 |
| Sex × Incisive strength | -0.145 | 1.092 | 62.999 | 0.132 | 0.895 |
| Age × Incisive strength | 0.048 | 0.023 | 58.322 | 2.132 | 0.037 |
| Sex × Age × Incisive strength | 0.029 | 0.058 | 57.548 | 0.507 | 0.614 |
| Molar Zinc content | Estimate | SE | <i>df.</i> | <i>t</i>-value | <i>P</i>-value |
| Intercept | 0.22 | 0.06 | 58.959 | 3.688 | <0.001 |
| Sex | 0.002 | 0.151 | 62.651 | 0.014 | 0.989 |
| Age | -0.004 | 0.003 | 59.916 | 1.128 | 0.264 |
| Molar strength | -0.285 | 0.14 | 55.731 | 2.037 | 0.046 |
| Sex × Age | -0.002 | 0.007 | 60.044 | 0.227 | 0.821 |
| Sex × Molar strength | 0.21 | 0.363 | 62.994 | 0.578 | 0.566 |
| Age × Molar strength | 0.03 | 0.011 | 61.449 | 2.711 | 0.009 |
| Sex × Age × Molar strength | -0.009 | 0.021 | 59.01 | 0.443 | 0.660 |

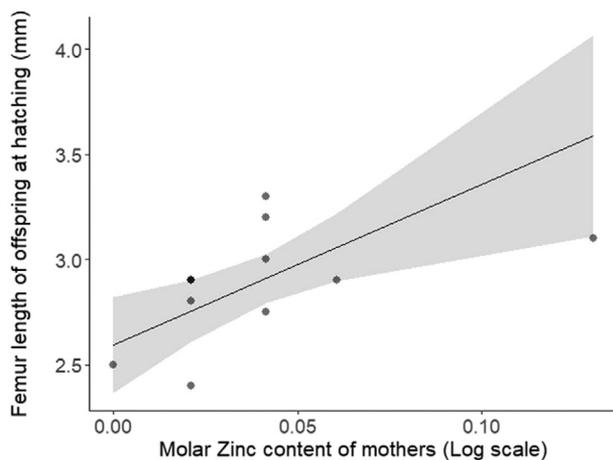


Figure 4. Relationship between the molar Zinc content of mothers and the body size of their offspring at hatching in *Chorthippus cazurroi*. The regression line and 95% confidence intervals after controlling for the effects of incisor Zinc content of mothers are also shown (see Table S6).

exhibiting the greatest force but not maximum Zinc enrichment (Figs. 1B and S3). Females with larger molar Zinc content produced slightly larger daughters at hatching (Fig. 4) but not at adulthood (Table S6; Fig. S4). It was adult body size that was more strongly correlated with mother's body size and that of the first instars was not (Table S6; Fig. S4). Incisor Zinc content of mothers was not associated with offspring size at any stage (all $t < 2.0$, $P > 0.07$).

EVOLUTION AND COEVOLUTION BETWEEN SEXES

The phylogenetic signal of mandibular Zinc content of species was low in incisors and molars of the two sexes. Blomberg's K was fully significant only in female molars ($K = 0.21$, $P = 0.030$), being nearly significant in male molars ($K = 0.17$, $P = 0.082$) and nonsignificant in incisors ($K = 0.07$ – 0.15 ; $0.11 < P < 0.81$). Lambda was not significantly different from zero in any mandible section and sex ($\lambda = 0$ – 0.42 , $0.20 < P < 1$). Uni- and multivariate tests analyzing the association among different combinations of variables revealed that incisors and molars variables significantly correlated among each other (Tables S7–S9) but, at the same time, unique and independent relationships emerged (e.g., between incisor strength and body size, or between molar strength and diet) (Table S7). For this independence, when mandible variables were grouped into blocks, few relationships remained significant (Table S8). We also found that environmental and mandible blocks were poorly associated if niche correlates were excluded from these combinations (Table S8). With this information, suggesting both coordinated and independent trajectories of variation, we built and tested for the reliability of a set of multivariate models that all included causal and direct relationships of environmental variables versus diet and body size, as well as simple correlations between mandible portions. These models differed in the number and type of links (causal or correlative) between diet, body size, strengths, and Zinc content. Figure 5 depicts the most plausible multivariate models identified by SEM. These showed that, in females more than in males, the evolution of mandible Zinc content was tied to that of

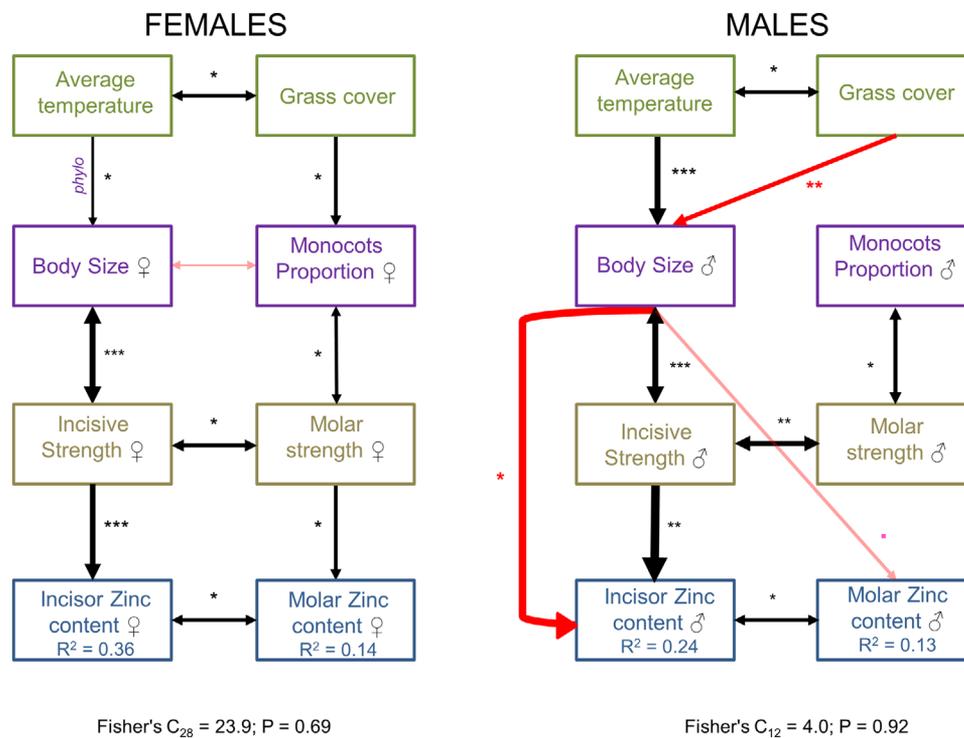


Figure 5. Direct and indirect pathways through which environmental variation (habitat and climate), body size, diet, and mandible strengths were linked to the differentiation of mandible Zinc content in Acrididae species, as highlighted by piecewise structural equation modeling. Female and male variation was analyzed separately, and only the best model of each sex is shown. Boxes represent measured variables, single head arrows represent causal relationships, and double head arrows covariation among variables. Black arrows, red arrows, and pink arrows denote, respectively, significant positive relationships, significant negative relationships, and nonsignificant negative relationships, included in these best models. Arrow thickness has been scaled based on the magnitude of standardised regression coefficients. The coefficients of determination for Zinc content are also shown. $P < 0.1$; * $P < 0.05$; ** $P < 0.01$, and *** $P < 0.001$. The term *phyl*o indicates relationships affected by phylogeny. The list of best models highlighted by structural equation modeling is presented in Table S10.

biomechanical properties, which in turn covaried with dietary habits (molar strength) and body size (incisors strength) of species. In particular, selection for stronger chewing mandibles in graminivorous species and the allometric increase of incisor strength with body size indirectly lead to enhanced Zinc content in female molars and incisors, respectively (Fig. 5A). The ultimate drivers of variation were temperature, slightly enhancing larger body size, and grassland cover, selecting for a specialized graminivorous diet. The above models for females poorly fit male data ($\Delta AIC = 44$) and the explanatory power of predictors on Zinc content in this sex was even lower than that of females ($r^2 = 0.13$ and 0.24 vs. 0.14 and 0.36 in molars and incisors of males and females, respectively) (Fig. 5). Male diet correlated with incisor strength, which in turn affected incisors Zinc content. Molars Zinc content was decoupled from biomechanics and was only weakly negatively influenced by body size. These models outperformed models including a direct relationship between ecological niche variables and Zinc content, or including phylogeny in paths

other than the environment-body size relationship (Fig. 5; Table S10).

As in the case of intraspecific analyses, Zinc content did not vary between sexes across species (paired *t*-tests in both mandible regions: $t_{28} < 1.0$, $P > 0.30$). Yet, male incisors Zinc did not track female one in species in which females were much larger, being correlated with that of females only in species with low sexual size dimorphism (phylogenetic GLS model: interaction term sexual dimorphism \times female Zinc content, Estimate = -5.96 ± 2.02 SE, $t_{25} = 2.94$, $P = 0.007$; phylogenetic signal $\lambda = 0.10$, nonsignificantly different from 0 [$P = 0.72$]). On the other hand, the molar Zinc content of males increased in concert with that of females (Estimate = 0.58 ± 0.18 ; $t_{27} = 3.11$, $P = 0.004$; $\lambda = 0$ [$P = 1$]) irrespective of the differences in size of the sexes ($P > 0.43$). This correlation implies that male molar Zinc content was significantly associated with female molar strength (Estimate = 7.28 ± 2.32 SE, $t_{28} = 3.13$; $P = 0.004$), whereas it was independent from male molar strength ($t_{28} = 1.12$; $P = 0.27$).

Discussion

Integrative approaches are required to obtain a complete picture of evolutionary change, which arises from variation among individuals, species, and higher clades. In this study, we found that mandibular Zinc content was repeatable among related individuals, was associated with cutting and chewing force and, in the molar region, was related with an indicator of fitness. These relationships suggest, with caution due the limitations of the experiment (see below), heritable variation, functional significance, and fitness consequences, that is, mechanisms that can bring about an adaptation process (Losos 1994; Lynch and Walsh 1998). However, there were also significant within and transgenerational environmental sources of resemblance, which may dampen the effects of natural selection and hinder evolutionary differentiation (Huey et al. 2003). These effects, along with interspecific correlations among traits and sexes, may explain the low predictive power of environmental factors in comparative analyses, but do not conceal the connection with the mandible biomechanical function.

The experiment with *Chorthippus cazurroi* showed that the relative content of Zinc in mandibles changed during development, mainly following ecdysis when some of the element was lost (Fig. 2). Nonetheless, the relative content in mature adults did not differ from that of newborn nymphs, in spite of a large increase in body size, which implies that Zinc had to be incorporated during growth. The magnitude of Zinc enrichment was related with that of mothers, was repeatable among adult full sibs after controlling for significant covariates (such as age for molar Zinc), and did not vary with sex, thus with the amount of food required and/or assimilated by individuals. This suggests that there might be an inherited threshold to the quantity of Zinc that an individual can incorporate. Concentrations dropped to a minimum in recently molted molars but they reached again the posthatching levels of family members in adult life. Notably, repeatability remained high among full-sib adults of the two periods (0.78–0.61), despite the significant shift in concentrations and the cross-sectional sample of these analyses (i.e., each individual was measured once). Clearly, these preliminary estimates of repeatability do not adequately approximate the (additive genetic) component of phenotypic variation that determines the response to selection (Lynch and Walsh 1998). An experiment with first generation individuals, even when parents also developed in the laboratory, may be unable to rule out transgenerational environmental effects (Mousseau and Dingle 1991). These were indeed found in this study: the significant influence of mother's age on mother-offspring correlation in molar Zinc content indicates a significant maternal effect at least in this mandibular region (Fig. 3).

In spite of the limitation of the experiment, however, this is the first study describing the ontogenesis of metal enrichment

during the different stages of an insect life cycle, and highlighting a significant family effect in this feature. There were within and transgenerational environmental effects, but irrespective of these, we also found distinctive thresholds of Zinc concentration maintained by full sibs up to death. This alleged signal of inheritance, which should be tested in further experiments with artificial and standardized food provision, is accompanied by evidence of adaptive molar Zinc enrichment. Mother's Zinc content correlated with offspring size at hatching (Fig. 4), a proxy of egg size that is crucial for survival at the beginning of grasshopper life cycle (Laiolo and Obeso 2015). Given the tight association of molar Zinc content with chewing power in mature adults (Table 1), it is likely that high levels of Zinc content improve long-term foraging efficiency, which in turn would enhance the amount or quality of resources allocated to eggs.

At the interspecific level, structural equation models disclosed how natural selection for functional performance translated into the evolution of species Zinc content. Selective forces imposed by the environment acted on mandible strengths, and mandible hardening evolved as a consequence of this selection (Fig. 5). Such evolutionary pathway was more plausible than alternative scenarios in which Zinc content was the direct target of environmental selection or evolved in parallel with a graminivorous diet. The lack of direct relationship with diet supports the observation that metal accumulation in insects is associated with an ample spectrum of food types. Monocots, which carry wearing silica bodies and opal phytoliths (Johnson 2011; Ibañez et al. 2013b), contributed to the evolution of harder and stronger mandibles in grasshoppers, but in other insects the same evolutionary outcome was produced by different food items, in general those offering great mechanical resistance (Polidori et al. 2020). This result implies that the magnitude and diversity of Zinc enrichment may only weakly predict the trophic diversification of clades.

Strong and hard mandibles also characterized large bodied grasshoppers, although in males there was also a negative direct correlation between body size and Zinc content. This result and other sexual differences, such as the determinants of body size, are likely associated with behavioral and physiological differences between sexes (Laiolo et al. 2013; Pato et al. 2019). The differential reproductive costs of the sexes may explain the stronger selective pressure exerted by the habitat and plant food in females, which consume vegetal biomass at a faster rate than males (Hewitt 1977). Furthermore, the significant intersexual correlation in the molar region, which links male Zinc to female strength, suggests a correlation between male traits and female fitness.

In conclusion, this study shows that Zinc enrichment is adaptive and its variation follows the changes of biomechanical properties, in turn induced by environmental conditions. However,

the coevolution with body size, the almost exclusive relationship with masticatory power, and the environmental sources of similarity among individuals are all factors that may limit the evolutionary opportunities for this trait. In contrast, many calcified organic tissues have evolved in media characterized by large environmental and functional opportunities, such as differences in environmental Calcium concentrations or multipurpose organs (feeding, defense, reproduction). These opportunities have contributed to fuel the diversification of calcified tissues (Carlson 1990; Knoll 2003). All in all, this study provides an example of the different factors that regulate the evolution of structural diversity in animals, and points out the importance of integrating evolutionary and functional approaches to disentangle the different steps of the evolutionary process.

AUTHOR CONTRIBUTIONS

PL, JRO, JP, and JCI conceived the ideas, designed the methodology, and collected the data. PL, JP, and JCI analyzed the data. PL led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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DATA ARCHIVING

Data are available in the Dryad repository (<https://doi.org/10.5061/dryad.3ffb79hk>)

CONFLICT OF INTEREST

The authors declare no conflict of interest

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Zinc-enrichment of mandible ridges.

Fig. S2. Phylogenetic relationships among Acrididae species from the Cantabrian Mountains.

Fig. S3. Relationships between Zinc-enrichment and mandible forces across ages in *Chorthippus cazurroi*.

Fig. S4. Relationship between the molar Zinc content (left) and body size (right) of mothers and the body size of their offspring at hatching (exuviae I) and adulthood in *Chorthippus cazurroi*.

Table S1. Relative elemental content of mandible ridges in grasshopper species from the Cantabrian Mountains.

Table S2. Reconstruction of phylogenetic relationships among species.

Table S3. Classification of grasshopper diet.

Table S4. Differences in the relative Zinc content of molars and incisors between stages of the life cycle of *Chorthippus cazurroi*.

Table S5. Effects of family identity, body size, age and egg pod order on the mandibular Zinc content of full-sib adults of *Chorthippus cazurroi*.

Table S6. Regression models testing for the relationship between offspring body size and the Zinc content in mandibles and body size of mothers.

Table S7. Results of phylogenetic generalized least square regressions testing for interspecific univariate relationships among Zinc content of incisors and molars, strengths of incisors and molars, body size, monocots proportion, temperature, and grass cover.

Table S8. Results of two-block partial least squares and multivariate phylogenetic least squares testing for interspecific multivariate relationships among different combinations of mandible variables, ecological niche correlates, and environmental factors.

Table S9. Results of phylogenetic generalized least square and multivariate phylogenetic least square models testing for the relationships between Zinc content (separately for molars and incisors, or jointly) versus the strengths of incisors and molars, body size, monocots proportion, temperature, and grass cover at the interspecific level.

Table S10. Piecewise structural equation models SEM testing for the relationships between mandibular Zinc content, mandibular strengths, and intrinsic and environmental predictors (body size, phylogeny, diet, climatic, and habitat variables)