Limited tolerance by insects to high temperatures across tropical elevational gradients and the implications of global warming for extinction

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The critical thermal maximum (CT_{max}), the temperature at which motor control is lost in animals, has the potential to determine if species will tolerate global warming. For insects, tolerance to high temperatures decreases with latitude, suggesting that similar patterns may exist along elevational gradients as well. This study explored how CT_{max} varies among species and populations of a group of diverse tropical insect herbivores, the rolled-leaf beetles, across both broad and narrow elevational gradients. Data from 6,948 field observations and 8,700 museum specimens were used to map the elevational distributions of rolled-leaf beetles on two mountains in Costa Rica. CT_{max} was determined for 1,252 individual beetles representing all populations across the gradients. Initial morphological identifications suggested a total of 26 species with populations at different elevations displaying contrasting upper thermal limits. However, compared with morphological identifications, DNA barcodes (cytochrome oxidase I) revealed significant cryptic species diversity. DNA barcodes identified 42 species and haplotypes across 11 species complexes. These 42 species displayed much narrower elevational distributions and values of CT_{max} than the 26 morphologically defined species. In general, species found at middle elevations and on mountaintops are less tolerant to high temperatures than species restricted to lowland habitats. Species with broad elevational distributions display high CT_{max} throughout their ranges. We found no significant phylogenetic signal in CT_{max}, geography, or elevational range. The narrow variance in CT_{max} values for most rolled-leaf beetles, especially high-elevation species, suggests that the risk of extinction of insects may be substantial under some projected rates of global warming.

Cephaloleia | *Chelobasis* | CO1 | CT_{max} | thermal limits

Tolerance of organisms to changing temperatures is a trait of broad interest from ecological, evolutionary, and conservation perspectives (1). Thermal limits determine the geographic distributions of organisms, local adaptations, and organismal responses to global warming (2, 3). The thermal adaptation hypothesis proposes that because adaptation to a broad range of temperatures has a high physiological cost, the thermal limits of populations and species will be selected to match temperatures that characterize their geographic ranges (2, 4). As a result, tolerance to high temperatures is assumed to be a conservative trait with limited phenotypic plasticity and evolvability (5, 6) [but an example of rapid evolution of CT_{max} is provided by Logan et al. (7)]. This limitation in phenotypic and evolutionary responses poses a serious threat to organisms under projected global warming (2).

In the tropics, environmental temperatures remain relatively constant through the year. However, the rate at which temperature decreases with elevation is highest near the equator [*ca.* 6.5 °C for 1 km in elevation (8)]. As a consequence, tropical biota display sharp transitions as life zones change with elevation. In ectotherms, such as insect herbivores, low temporal but high spatial variation in temperatures along tropical mountains is expected to select for narrow thermal limits (2, 3, 9). A central prediction of the thermal adaptation hypothesis is that tropical insects living in the lowlands

will be more tolerant to high temperatures than species at higher elevations (10). One potential exception for this pattern is the ectotherms present in tropical alpine ecosystems, which will experience extreme high and low temperatures during the day and night (11). Species distributions are not necessarily restricted to one discrete life zone, and it is unclear how tolerance to high temperatures changes among insect populations for species present at multiple elevations.

To determine how tolerance of insect herbivores to high temperatures varies along elevational gradients, the following biogeographic, taxonomic, and physiological information should be considered. First, accurate estimates of altitudinal distributions of insect herbivore species are needed. Unfortunately, records of elevational distributions for most tropical insects are nonexistent or at best fragmentary (12). Second, because a species is the unit of analysis, the underpinning taxonomy must be stable, well supported, and with clear species boundaries. Unfortunately, only 0.3–1.2% of an estimated 8.7–30 million insect species on earth have been described so far (13). Molecular tools, such as DNA barcoding, are revealing even broader gaps in our taxonomic knowledge, because it is routine to discover that well-established species are actually complexes of multiple cryptic species (14).

The third challenge is to estimate the physiological tolerance to high temperatures of insect herbivores. A standard laboratory method is to measure the critical thermal maximum (CT_{max}), the temperature at which insect herbivores lose motor control when exposed to high temperatures (15). CT_{max} estimates are highly dependent on experimental conditions, such as initial ambient

Significance

Tolerance to high temperatures will determine the survival of animal species under projected global warming. Surprisingly little research has been conducted to elucidate how this trait changes in organisms living at different elevations of similar latitudes, especially in the tropics. DNA barcodes demonstrate that insect species previously thought to have broad elevational distributions and phenotypically plastic thermal tolerances actually comprise cryptic species complexes. These cryptic species occupy discrete elevational ranges, and their thermal tolerances seem to be locally adapted to temperatures in their life zones. The combination of high species endemism and local adaptation to temperature regimes may increase the extinction risk of high-elevation insects in a warming world.

temperatures and rates of temperature increase (16). This dependency has raised some doubt about the validity of previous studies comparing CT_{max} using different methods (16).

The central objective of this research is to understand how tolerance to high temperatures differs among insect species with narrow vs. broad elevational distributions based on detailed distribution records with the identification of cryptic species using DNA barcodes. For species with narrow elevational distributions, we expect that species inhabiting warmer regions (i.e., warmer elevational gradients or life zones in the lowlands) would display higher CT_{max} values than species inhabiting cooler environments.

We are particularly interested in understanding how CT_{max} changes in species with broad elevational distributions, because these species provide an opportunity to determine if ectotherm tolerance to high temperatures is a labile or fixed character. If populations at different elevations display the same CT_{max} despite changes in environmental temperatures, this similarity will suggest that CT_{max} is a fixed trait that may limit the colonization of environments with different temperatures. If populations of the same species inhabiting different elevations display different CT_{max} values, then two different scenarios are possible. One scenario would be that CT_{max} is a trait with more phenotypic plasticity than previously suggested by the thermal adaptation hypothesis. The second scenario is that populations along elevational gradients represent locally adapted genotypes or even cryptic species. Our study explores the roles of phenotypic plasticity or local adaptation under these two scenarios using molecular markers combined with extensive field records and laboratory experiments.

To determine how insect herbivore tolerance to high temperatures differs among species and populations inhabiting different elevations along tropical elevational gradients, we combined (*i*) extensive field and museum records of insect herbivore distributions along elevational gradients with (*ii*) the identification of cryptic taxa using DNA barcodes and (*iii*) CT_{max} estimates that were recorded using standardized methods.

We performed this study in Costa Rica, Central America, along two elevational gradients: (*i*) the Barva Volcano gradient located near the north-Atlantic border with Nicaragua and (*ii*) the Talamanca elevational gradient near the south-Pacific border with Panama. For both elevational gradients, lowland tropical rain forests transition to premontane and montane forests at higher elevations. Monthly temperatures on the Talamanca transect are 1–3 °C warmer than at equivalent life zones on the Barva transect (Fig. S1 and Table S1).

For our investigation, we selected a diverse group of well-known tropical insect herbivores, the rolled-leaf beetles (genera *Cephaloleia* and *Chelobasis*; family Chrysomelidae). Rolled-leaf beetles complete their life cycles inside the scrolls formed by the young leaves of their host plants, species in the order Zingiberales (17).

Leaf transpiration keeps relative humidity constant inside rolled leaves (close to 100%) as external environmental humidity fluctuates throughout the day (Fig. S2). However, temperatures inside the rolled-leaf scrolls are always very close to the air temperature surrounding the host plant (Fig. S2). Because the full life cycles of rolled-leaf beetles occur within young rolled leaves of their host plants and beetles exhibit no evidence of thermoregulatory behavior, the operative temperatures of these beetles, (i.e., steady-state body temperatures) are very close to ambient air temperatures (2).

We used a large dataset of 6,948 field and 8,700 museum records to determine the elevational distributions of what traditional morphological methods identified as a total of 26 rolled-leaf beetle species distributed along two elevational gradients. However, after delimiting species using the DNA barcode cytochrome oxidase I (CO1), we identified a total of 42 rolled-leaf beetle species and haplotypes distributed among 54 populations. For each population, we assessed beetle tolerance to high temperatures (CT_{max}) using standardized methodologies (*Materials and Methods*). After estimating CT_{max} for 1,252 beetles, we

amplified the DNA barcode CO1 for each individual. Sequences were used for further analyses of genetic structure among populations at different elevations and for the identification of cryptic species (Fig. S3).

Results

Phylogenetic Signal of CT_{max}, Geographic, and Insect Altitudinal Distributions. A potential issue of selecting closely related species for this study is the possibility of phylogenetic pseudoreplication. One example of the potential effects of phylogenetic-induced bias in our analyses is a scenario where species living in the same life zone or at the same elevational gradient are closely related (18). If species within the same life zone or elevational gradient distribution display similar thermal limits, these similarities might be the product of evolutionary history, not local adaptation.

To explore any potential effect of phylogenetic pseudoreplication in this study, we tested for phylogenetic signals associated with geographic origin (elevational gradient), altitudinal distribution (life zone), and thermal limits (mean CT_{max}). We assembled a phylogenetic tree using Bayesian inference methods. We tested for the presence of phylogenetic signal using Blomberg's K (package Picante, Program R 3.0.2) (19). We detected phylogenetic overdispersion in geographic distributions. Closely related taxa tend to be present in different cordilleras (K = 0.13, *P* PIC.variance = 0.01). We did not detect phylogenetic signal in elevational distributions of rolled-leaf beetles along elevational gradients (K = 0.03, *P* PIC.
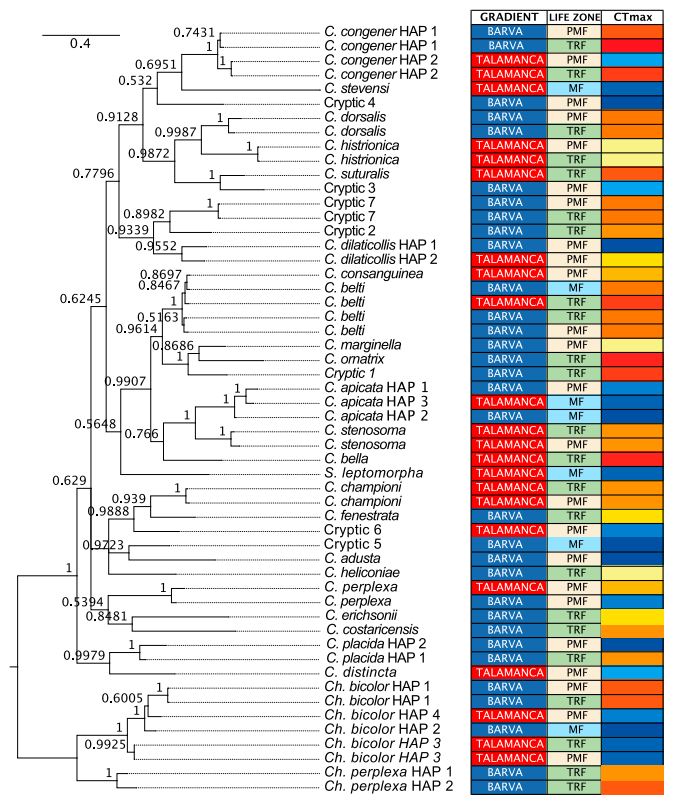

Fig. 1. Testing for phylogenetic signal in the geographic distributions (elevational gradient), elevational distributions (life zone), and upper thermal limits (CT_{max}) of rolled-leaf beetles. Phylogenetic relationships among populations, haplotypes, and beetle species were inferred using CO1 sequences. Node values represent posterior probabilities. Geographic origin, altitudinal distribution, and mean CT_{max} of each clade are represented in the table adjacent to the tips. There is no evidence of phylogenetic pseudoreplication affecting our analyses. We detected phylogenetic overdispersion in geographic distributions and upper thermal limits (CT_{max}). No phylogenetic signal was detected for elevational distributions along elevational gradients. The CT_{max} scale is as in Fig. 3.

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variance = 0.7). We detected phylogenetic overdispersion in CT_{max}. Closely related taxa tend to display different CT_{max} values (K = 0.11, P PIC.variance = 0.01). These results suggest no effect of phylogenetic pseudoreplication in the results of this study (Fig. 1).

Cryptic Species Diversity of Insect Herbivores Along Elevational Gradients. In addition to identifying all obvious morphologically distinct rolled-leaf beetle taxa with 100% accuracy, the DNA barcode CO1 recognized several cryptic species (Fig. 2). We also detected a second DNA barcode gap for 16 populations representing six insect herbivore species. In these cases, differences between haplotypes were between 90% and 95% (Fig. 1). These cases may represent population differentiation or events of incipient speciation. To be conservative, we suggest that these clades represent different CO1 haplotypes within the same species. The methods used to delimit species and haplotypes are described in Fig. S3.

Based on 1,184 CO1 DNA sequences from 42 species and haplotypes, collected from 54 populations along two elevational gradients (Fig. 2), we discovered a total of 11 species complexes (Fig. 3). More than 50% of all taxa in this study are part of a cryptic species/haplotype complex (Fig. 3). When cryptic taxa within the same taxonomic complex were present at different life zones, high-elevation taxa displayed lower CT_{max} values than those taxa at lower elevations (Fig. 3).

CT_{max} of Insect Herbivores Are Higher Along the Warmer Elevational Gradient. When comparing the CT_{max} of insect herbivores at equivalent life zones on the Barva and Talamanca transects, as predicted by the thermal tolerance hypothesis, the CT_{max} of insect herbivores was always higher for species on the warmer Talamanca gradient ($F_{elevational\ gradient}$ = 89.3, df = 1, P > 0.001; $F_{life\ zone}$ = 712.1, df = 2, P > 0.001; monthly temperature variation for both elevational gradients are shown in Fig. S1 and Table S1). On average, the CT_{max} of the lowland forest insects at Talamanca was 1.7 °C higher than on the Barva transect (mean ± SD_{Barva} = 42.7 ± 1.1 °C, mean ± $SD_{Talamanca}$ = 44.4 ± 0.77 °C; Fig. 4). The CT_{max} of insect herbivores in premontane forests on the
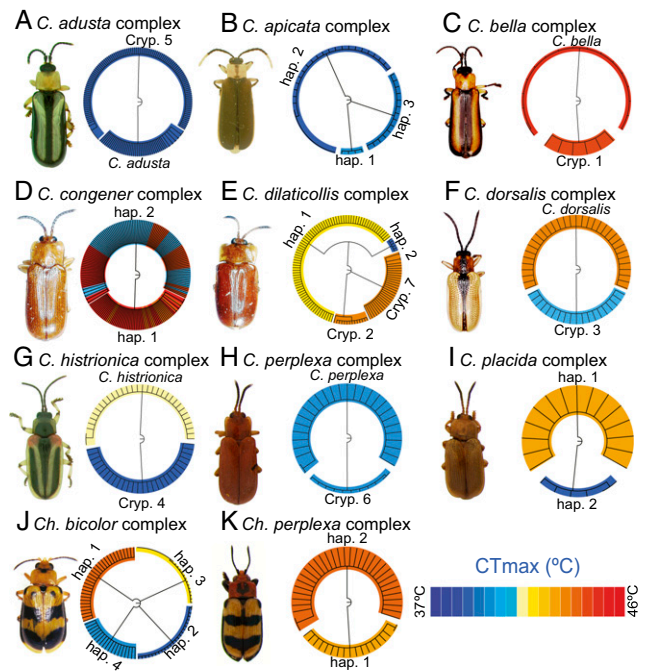

Fig. 3. Rolled-leaf beetle haplotypes and cryptic species complexes on the Barva (north) and Talamanca (south) elevational gradients (*A–K*). Neighbor-joining tree branches group different haplotypes and cryptic species, with all branch support values = 100. Colors represent the CT_{max} of each taxon. Elevational distributions are shown in Fig. 6.

Talamanca transect was 2.3 °C higher than the CT_{max} of insects at the same life zone on the Barva transect (mean ± SD_{Barva} = 39.2 ± 1.3 °C, mean ± $SD_{Talamanca}$ = 41.5 ± 1.09 °C; Fig. 4). Insect herbivores inhabiting the Talamanca montane forest displayed an average CT_{max} 1.5 °C higher than the CT_{max} of insect herbivores in the same life zone on the Barva transect (mean ± SD_{Barva} = 37.3 ± 1.8 °C, mean ± $SD_{Talamanca}$ = 38.8 ± 1.7 °C; Fig. 4).

CT_{max} of Insect Herbivores with Narrow Elevational Distributions Decreases with Increasing Elevation. As predicted by the thermal tolerance hypothesis, the CT_{max} of insect herbivore taxa present at only one life zone decreases with increasing elevation (Fig. 4). On both elevational gradients, herbivores in the lowland forests had the highest CT_{max} (Fig. 4). Species in premontane forests had lower CT_{max} values than lowland taxa (Fig. 4), whereas the lowest CT_{max} values were recorded in species present only at the highest elevations of each gradient, in montane forests ($F_{20,2}$ = 64.6, 513.1, $P_{life\ zone}$ < 0.0001, $P_{species}$ < 0.0001; Fig. 4).

CT_{max} of Insect Herbivores Represented by One Haplotype at Multiple Life Zones Remains Constant with Increasing Elevation. In general, when beetle species represented by a single haplotype were present in multiple life zones along a gradient, CT_{max} remained constant among all populations along the species' elevational range (Fig. 5 *A*, *B*, and *E–H* and Fig. 6). This pattern is a potential consequence of higher gene flow across populations.

We found only one exception to this pattern. Although *Cephaloleia congener* is represented by one broadly distributed haplotype, high-elevation populations of *C. congener* on the Barva and Talamanca elevational gradients displayed CT_{max} values 1.3 °C and 3.9 °C lower than populations in the lowlands (Fig. 5 *C* and *D*). It is possible that this species has a unique genetic difference of thermal limits. However, we suspect that this pattern may represent a particular case where DNA barcodes were not able to differentiate haplotypes, because high-elevation populations of
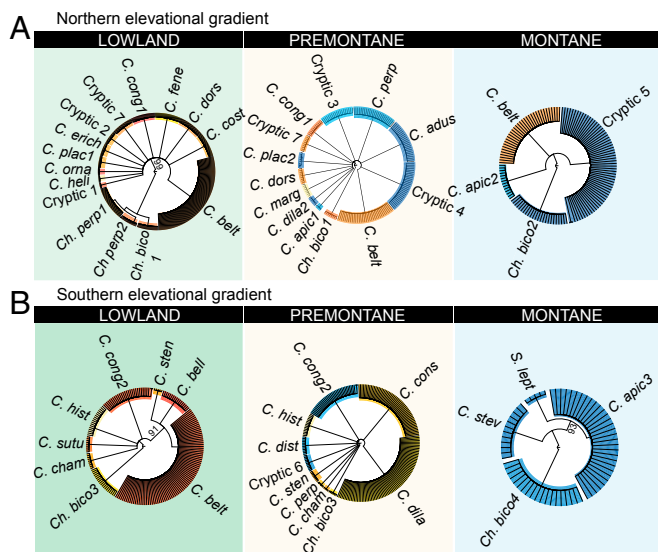

Fig. 2. Rolled-leaf beetle species and haplotype distributions along two elevational gradients. Barva transect (north) (*A*) and Talamanca cordillera (south) (*B*). Species and haplotype identifications are based on neighbor-joining trees after alignment of DNA CO1 sequences, with all branch support values = 100 (unless otherwise noted). Beetle species are abbreviated using the first four letters of species names. Full names are provided in Fig. 1. Colors represent the CT_{max} of beetle populations in each life zone as in Fig. 3. Elevational distributions are shown in Fig. 6.
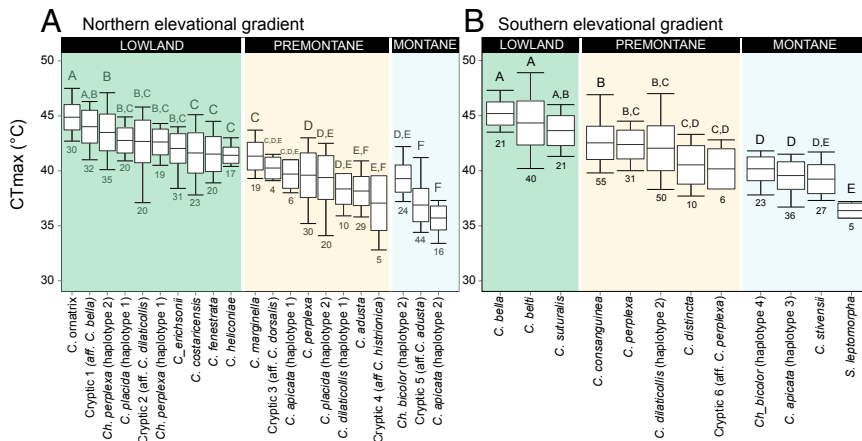
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Fig. 4. CT_{max} of rolled leaf beetle taxa restricted to one life zone (mean ± SD, minimum–maximum). Barva (north) (*A*) and Talamanca (south) (*B*) elevational gradients. Numbers below boxes represent sample sizes. Letters on the bars group similar categories.

C. congener display some unique morphological features compared with lowland populations.

CT_{max} of Insect Herbivores Represented by Multiple Haplotypes Along Elevational Gradients Decreases with Increasing Elevation. For cases in which beetle species were represented by multiple CO1 haplotypes at different elevations, high-elevation haplotypes displayed CT_{max} values lower than in lowland populations (Fig. 5 *I–L*). High-elevation beetle haplotypes are characterized by low thermal limits; this pattern supports the hypothesis that populations along elevational gradients are locally adapted genotypes.

Discussion

Our results support the hypothesis that in wild populations of ectotherms, tolerance to high temperatures is a trait with limited phenotypic plasticity. As predicted by the thermal limitation hypothesis, these results show that the CT_{max} of insect species is higher along warmer elevational gradients and decreases with increasing elevation (10). Limited phenotypic plasticity and evolvability in CT_{max} have also been reported for model organisms, such as *Drosophila*, the tsetse fly *Glossina pallidipes*, and some dung beetle species (16, 20, 21).

Fig. 5. CT_{max} of rolled beetle species represented by one or more haplotypes present at multiple life zones (*A–L*; mean ± SD, minimum–maximum). Numbers below boxes represent sample sizes.

INSECT HAPLOTYPES AT DIFFERENT ELEVATIONS

These results are in contrast to the conclusions of systematic reviews of global empirical data (i.e., studies that compile CT_{max} estimates for studies that used different methods and multiple taxa) (2, 4). Compilation analyses often report a slight decline or even no relationship between arthropod elevational distributions and CT_{max} (2, 20). Some of the mechanisms invoked to explain this discrepancy with the thermal limitation hypothesis include competitive exclusion, predation, behavioral thermoregulation, and the absence of other interacting species (4, 20, 22).

Biotic factors and behaviors may generate a mismatch between ambient temperatures and the thermal limits of organisms. However, our results suggest that this discrepancy may also be an artifact of combining data from studies that used nonstandardized methods, a fragmentary knowledge of insect elevational distributions, and limited taxonomic resolution (23). These three factors greatly reduce the reliability of synthetic studies to find patterns at fine geographic and taxonomic scales. For example, if this study had determined the CT_{max} of only high-elevation rolled-leaf beetle populations, ignoring cases that represent high-elevation populations of broadly distributed species (e.g., high-elevation populations of *Cephaloleia belti*, *Cephaloleia championi*, *Cephaloleia dorsalis*, *Cephaloleia stenosoma*, Cryptic sp. 7, *Chelobasis bicolor* hap1; Fig. 6), we would mistakenly conclude that high-elevation species often display CT_{max} values similar to species in the lowlands.

This study also illustrates the importance of accurate species delimitations to understand the thermal limits of insects (23). Traditional morphological taxonomic approaches would assume that each cryptic species complex included in this study is a single broadly distributed species (17) (Fig. 3). Without the insight provided by DNA barcodes, we would wrongly conclude that CT_{max} is a trait that varies with elevation. Our results show an opposite scenario, where CT_{max} is a trait that is apparently fixed and characteristic to each species and haplotype (Fig. 3 *E–K*).

It is expected that after a temperature increase of 3–6 °C, which is predicted for the next century, isotherms along the Barva and Talamanca gradients will shift *ca.* 600 m upslope (24). Therefore, insect populations present at the highest elevations also face the highest risks of extinction as their thermal habitat disappears and they are "pushed" off the tops of these mountains (24). Our DNA barcode data revealed an even more critical scenario in which most of the high-elevation insect populations are actually endemic cryptic species complexes with very narrow elevational distributions.

As the climate changes, the persistence of insect herbivore populations will initially depend on their dispersal abilities and, in the long term, on the heritability of traits associated with adaptations to novel temperatures (25). More and more evidence suggests that CT_{max} in insects is an evolutionarily constrained trait with
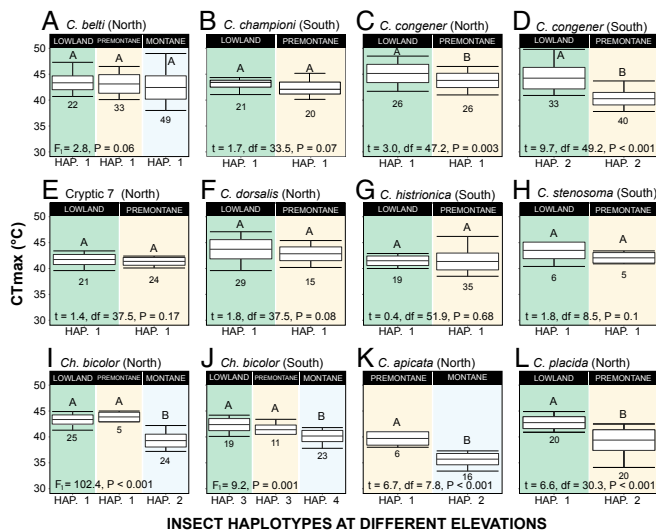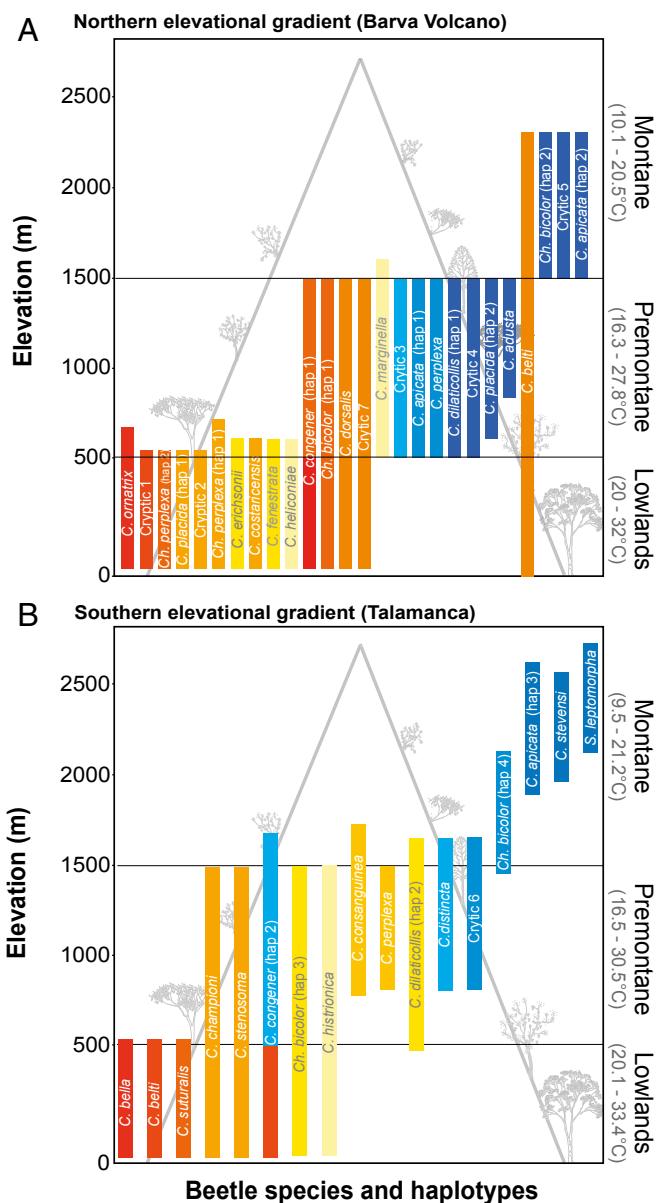
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Fig. 6. Reconstructing the maximum thermal limits of rolled-leaf beetles along elevational gradients. Barva transect (north) (*A*) and Talamanca elevational gradient (south) (*B*). Bars represent the elevational distributions of rolled-leaf beetle species along elevational gradients. Temperatures on the right *y* axis represent the minimum–maximum air temperatures potentially experienced by rolled-leaf beetles throughout the year (37). The CT_{max} scale is as in Fig. 3. A detailed description of minimum–maximum average temperatures throughout the year at each life zone is included in Table S1.

Unfortunately, we do not yet know if rolled-leaf beetles will be able to adapt rapidly, in ecological time, to mitigate the effects of rapid global warming.

Evidence suggests that ectotherms can rapidly acclimate and adapt to cold temperatures. However, adaptation to warmer temperatures seems to be more challenging (21, 29). For example, after only a few generations, *Anolis* lizards and sticklebacks are able to adapt to colder, but not to warmer, environments (5, 6). This pattern of high evolvability of critical thermal minimum but low potential for adaptation of CT_{max} has also been described for several endo- and ectotherms (30). Adaptation to warmer temperatures in ecological time is also possible; there is evidence of rapid evolution of thermal tolerance to warm temperatures in anurans and lizards (7, 31).

Further studies on the potential for adaptation to novel conditions are vital to understanding the complex effects of global warming on the persistence of insect populations along elevational gradients. Habitat loss and thermal constraints limiting the colonization of warmer environments are two main challenges that insects will confront under projected global warming.

Materials and Methods

Study Site and Species. This study was conducted on the Barva transect (eastern slope of the Central cordillera: 10° 0′–10° 26′ N, 83° 59′–84° 07′ W) and the western slope of the Talamanca cordillera (8° 38′–8° 58′ N, 82° 50′–83° 05′ W) in Costa Rica, Central America. The Barva transect, on the north-Atlantic slope of Costa Rica near the border with Nicaragua, extends from 50 to 2,800 meters above sea level (m.a.s.l.). We collected rolled-leaf beetles from the tropical rain forests of La Selva Biological Station and near shelters in the connected Braulio Carrillo National Park from premontane (700 and 1,070 m.a.s.l.) and montane (1,500 and 2,100 m.a.s.l.) forests.

Our study area on the Pacific slope in the Talamanca cordillera includes tropical rain forests in the lowlands from 60–400 m.a.s.l. surrounding the localities of Ciudad Neily to Fila de Cal, premontante forest at 1,200 m.a.s.l. within Las Cruces Biological Station and surrounding forest fragments, and the montane forests at Parque Nacional La Amistad that extend from 1,500 to 2,100 m.a.s.l.

Minimum temperatures along both elevational gradients are similar throughout the year (Fig. S1 and Table S1). However, monthly maximum temperatures in the Talamanca cordillera are 1–3 °C warmer than at equivalent life zones along the Barva transect (Fig. S1 and Table S1).

Recording Elevational Distributions of Insect Herbivores. To determine the elevational distributions of rolled-leaf beetles along these two elevational gradients, we used a large dataset of rolled-leaf beetles collected along the two gradients since 2005. Elevational distributions of rolled-leaf beetles are based on morphological identifications of 6,948 field records and the revision of 8,700 museum specimens from Costa Rica. Elevational distribution records were included in two monographs of the genera *Cephaloleia* and *Chelobasis* (17, 32). Vouchers were deposited in the Entomological Collection, National Museum of Natural History, Smithsonian Institution, and Instituto Nacional de Biodiversidad (INBio) in Costa Rica.

Phylogenetic Signal of CT_{max}, Geographic, and Altitudinal Distributions. To determine the effect of potential phylogenetic pseudoreplication on thermal limits analyses, we assembled a phylogenetic tree representing phylogenetic relationships among populations, haplotypes, and beetle species. CO1 sequences representing an individual from each rolled-leaf beetle population included in this study were aligned using MAFFT V.7 (33). We generated a fully resolved tree using Bayesian inference methods in Mr. Bayes 3.21 (substitution model: HKY85, chain length: 500,000). The presence of phylogenetic signal was tested using Blomberg's K (package Picante, Program R 3.0.2) (19).

Estimating CT_{max} for Rolled-Leaf Beetle Populations. To determine how CT_{max} changes along elevational gradients for species of rolled-leaf beetles, we measured the temperature tolerances of 1,252 live beetles, representing 42 species and haplotypes distributed among 54 populations (sample size is shown in Figs. 4 and 5). For this study, beetles were directly collected from the field and kept in a temperature-controlled laboratory. Beetles were fed ad libitum and acclimated to 23 °C (a temperature experienced by beetles at each elevation on a daily basis) for 24 h before the start of experiments.

In an additional experiment, we tested the effect of acclimation time on CT_{max} estimates reported in this study. We found that CT_{max} estimates are

limited evolvability (21, 26, 27). Adaptation to novel temperatures will be especially crucial for high-elevation insect herbivores, because they display the lowest tolerance to high temperatures.

A crucial question is if the CT_{max} trait displays the evolvability required to respond to global warming. In evolutionary time, rolled-leaf beetles were able to colonize and adapt to local temperatures, as suggested by this study. Phylogenetic analyses showed that closely related species display contrasting CT_{max} values (Fig. 1). This phylogenetic repulsion suggests multiple scenarios regarding the potential for local adaptation after the colonization of novel environments. This result contrasts with phylogenetic analyses for 94 species of *Drosophila*, where heat resistance was dictated by phylogenetic relationships, rather than local adaptation (28).

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equivalent after 24–72 h of acclimation; however, long acclimation times have a negative effect on the physiological condition of rolled-leaf beetles, inducing high mortality in some species (Table S2).

To measure CT_{max}, we used a water bath with a volume of 1,000 cm^3 controlled by a thermostat (−50 to 110 °C, liquid crystal display Digital Temperature Measuring Controller WH7016M l, Lerway Technology). Beetles were placed in individual Falcon 15-mL Conical Centrifuge Tubes. Tubes were placed horizontally, floating on the surface of the water bath. Temperature sensors were placed inside the tubes. Therefore, temperatures reported in this study are those temperatures inside the 15-mL centrifuge tubes, not the basal temperatures of beetles used in the experiments. To simulate the high-humidity conditions inside rolled leaves, and to ensure that insect responses were elicited by changes in temperature and not desiccation, we placed a 1 × 3-cm wet filter paper inside each tube. Tubes with beetles were placed inside the water bath at an initial temperature of 23 °C.

Temperature increased at a rate of 1.5 °C·min^{-1}, with an accuracy of ±0.1 °C. As the temperature increased, tubes were rotated so that beetles were constantly walking along the bottom of the tubes, close to the water. As the temperature increased, beetles became more active until, reaching a temperature at which they lost muscular control, they flipped over onto their dorsa and ceased walking (knockdown temperature). We used these knockdown temperatures as an approximation of the CT_{max} for rolled-leaf beetle populations and species.

Knockdown temperatures depend on the temperature increase rate selected for the experiment (16). In this study, we selected an increase rate of 1.5 °C·min^{-1}. This rate is at least one order of magnitude faster than any temperature increase experienced by insects under natural conditions. We want to emphasize that the CT_{max} estimates reported in this study represent the relative resistance to high temperatures of rolled-leaf beetle populations and species, not the actual ambient temperatures at which populations will become locally extinct.

Identification of Cryptic Species and Haplotypes. Cryptic species and haplotypes of rolled-leaf beetles were identified using the DNA barcode CO1. Over the past decade, numerous studies have demonstrated that this molecular marker can delimit species with extreme accuracy in many taxa (14). This DNA barcode is particularly effective in the identification of rolled-leaf beetle species. In previous studies, we were able to identify all morphologically distinct species of rolled-leaf beetles with 100% accuracy (14, 34). After measuring CT_{max}, we collected beetles in 95% (vol/vol) ET-OH. One leg was subsequently removed for DNA extractions following the protocols described by García-Robledo et al. (34). Sequences were aligned using MAFFT (35). DNA sequences were deposited in the GenBank (accession nos. KU357054–KU358485). A description of the methods used to delimit cryptic species and haplotypes is included in Fig. S3.

Estimation of Differences in CT_{max} Between Taxa at Different Life Zones. Differences in CT_{max} among beetle species present only at one life zone were estimated using fully crossed ANOVA. The model included elevational gradients, life zones, sex, and beetle taxa as factors. The response variable was the CT_{max} of each individual. There was no effect of sex (male vs. female) on CT_{max} estimates for any of the species ($F_1 = 0.49$, $P = 0.48$). To determine if beetle species at different elevations display differences in size, potentially affecting the results reported in this study, we measured lengths for a subset of individuals of the species included in this study. We performed a regression between elevation (meters) and the average length per species of individuals found at a given elevation. We did not detect a relationship between elevation and beetle size ($n = 779$, $F_1 = 0.12$, $P = 0.7$). Therefore, the results reported in this study cannot be attributed to differences in size among species at different elevations.

For beetle species with broad elevational distributions and populations at two or more life zones, we tested for differences in CT_{max} among populations using ANOVA analyses or Aspin–Welch tests (36). Models included life zone as a main factor, and the response was the CT_{max} of each individual in each population. Differences among species and populations were determined with a posteriori tests (Tukey honest significance difference). All analyses were performed using Program R (19).

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