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

Carlos Garcia-Robledo<sup>a,b,1</sup>, Erin K. Kuprewicz<sup>2</sup>, Charles L. Staines<sup>b</sup>, Terry L. Erwin<sup>3</sup>, and W. John Kress<sup>a</sup>

<sup>a</sup>Department of Botany, National Museum of Natural History, Smithsonian Institution, Washington, DC 20013-7012; and <sup>b</sup>Department of Entomology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20013-7012

Edited by Douglas Futuyma, and approved November 24, 2015 (received for review April 20, 2015)

The critical thermal maximum (CT<sub>max</sub>), 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<sub>max</sub> 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<sub>max</sub> 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<sub>max</sub> 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<sub>max</sub> throughout their ranges. We found no significant phylogenetic signal in CT<sub>max</sub> geography, or elevational range. The narrow variance in CT<sub>max</sub> 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 | COI | CT<sub>max</sub> | 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<sub>max</sub> is provided by Logn 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<sub>max</sub>), the temperature at which insect herbivores lose motor control when exposed to high temperatures (15). CT<sub>max</sub> 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.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The DNA sequences reported in this paper have been deposited in the GenBank database, dx.doi.org/10.5883/DS-BOFCR (accession nos. KU357054–KU358485).

1To whom correspondence should be addressed. Email: garciac@si.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1507681113/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1507681113

PNAS Early Edition | 1 of 6
temperatures and rates of temperature increase (16). This dependence has raised some doubt about the validity of previous studies comparing $C_{T_{\text{max}}}$ 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 $C_{T_{\text{max}}}$ values than species inhabiting cooler environments.

We are particularly interested in understanding how $C_{T_{\text{max}}}$ 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 $C_{T_{\text{max}}}$ despite changes in environmental temperatures, this similarity will suggest that $C_{T_{\text{max}}}$ 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 $C_{T_{\text{max}}}$ values, then two different scenarios are possible. One scenario would be that $C_{T_{\text{max}}}$ 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) $C_{T_{\text{max}}}$ 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 (genus *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 ($C_{T_{\text{max}}}$) using standardized methodologies (*Materials and Methods*). After estimating $C_{T_{\text{max}}}$ 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 $C_{T_{\text{max}}}$ 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 ($C_{T_{\text{max}}}$). 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 ($K = 0.13$, $P_{\text{PIC}-\text{variance}} = 0.01$). We did not detect phylogenetic signal in elevational distributions of rolled-leaf beetles along elevational gradients ($K = 0.03$, $P_{\text{PIC}}$).
variance = 0.7). We detected phylogenetic overdispersion in CT_{max}. Closely related taxa tend to display different CT_{max} values (K = 0.11, P = 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

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 were 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
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 A–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).

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 non-standardized 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
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 $CT_{\text{max}}$ 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 $CT_{\text{max}}$ 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).

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 $CT_{\text{max}}$ 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° 6′ – 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, premontane 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 $CT_{\text{max}}$, 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.7.3 (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 Blumberg’s K (package Picante, Program R 3.0.2) (19).

**Estimating $CT_{\text{max}}$ for Rolled-Leaf Beetle Populations.** To determine how $CT_{\text{max}}$ 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 $CT_{\text{max}}$ estimates reported in this study. We found that $CT_{\text{max}}$ estimates are
for rolled-leaf Nucleic Acids Res = Biol J Linn Soc J Exp Biol = among populations estimates for any of the species (Proc Natl Acad Sci USA Coleopt Bull = We thank D. J. Futuyma and two anonymous reviewers of each individual. There was no effect of sex (male vs. female) on Chevrolat, 1836 (Co-
want to emphasize that the CT temperature at which they lost muscular control, they flipped over onto As the temperature increased, beetles became more active until, reaching a humidity conditions inside rolled leaves, and to ensure that insect responses temperatures of beetles used in the experiments. To simulate the high-
controlled by a thermostat (Proc Natl Acad Sci USA
15. Lutterschmidt WI, Hutchison VH (1997) The critical thermal maximum: History and
critique. 35. Biomatters-Development-Team (2012) Geneious Pro 5.6.5 (Biomatters, Auckland).

species of rolled-leaf beetles with 100% accuracy (14, 34). After measuring CTmax, 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 in-
cluded in Fig. S3.

Estimation of Differences in \( CT_{\text{max}} \) Between Taxa at Different Life Zones. Differences in \( CT_{\text{max}} \) among beetle species present only at one life zone were estimated using fully crossed ANOVA. The model included elevational gra-
dients, life zones, sex, and beetle taxa as factors. The response variable was the \( CT_{\text{max}} \) of each individual. There was no effect of sex (male vs. female) on \( CT_{\text{max}} \) estimates for any of the species (\( F_1,0.49 = 0.48 \)). 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 re-
gression 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, F1,0.12 = 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 \( CT_{\text{max}} \) among populations using ANOVA analyses or Aspin–Welch tests (36). Models included life zone as a main factor, and the response was the \( CT_{\text{max}} \) of each individual in each population. Differences among species were also tested using \( F \)-tests with an a posteriori test (Tukey honest significance difference). All analyses were performed using Program R (19).

ACKNOWLEDGMENTS. We thank D. J. Futuyma and two anonymous reviewers for their constructive suggestions. We thank M. Kuzmina and C. Puente for assistance with DNA barcode generation and analyses. This study was supported by grants from the Smithsonian Institution Postdoctoral Fellowship Program, the Global Earth Observatories Program, the Office of the Under Secretary for Science, National Geographic/Walton Institute Grant W149-11 (to C.G.-R.), Smith-