



RESEARCH ARTICLE - BEES

Thermal Tolerance of Honeybees (*Apis mellifera* L.) Changes Across an Elevation Gradient in the Mexican Transition Zone

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Abstract

The objective of this study was to determine the critical thermal minimum [CTmin], critical thermal maximum [CTmax], and thermal tolerance range of *A. mellifera* at three different elevations located in the Mexican Transition Zone: 11; 1,324, and 3,304 m.a.s.l. In general, we found that the CTmin of *A. mellifera* was lower at the site with the highest elevation (i.e., they tolerate colder temperatures). At the same time, the CTmax remained constant across the three studied elevations, revealing higher plasticity for cold tolerance rather than heat. Moreover, we did not find evidence that the body mass of the individuals was associated with their thermal tolerance at any of the three sampled elevations. Our findings suggest processes of local adaptation of *A. mellifera* populations in environmentally contrasting sites, allowing them to expand their range of distribution, which could be useful in predicting responses to future environmental change.

Introduction

Current and historical temperatures are considered the main factors shaping the geographic distribution and abundance of life on Earth (Bennett et al., 2021) because temperature changes can negatively affect organisms if they exceed their thermal tolerance limits (Chown & Nicolson, 2004). Insects are extremely vulnerable to environmental changes as they are highly dependent on external temperature to ensure their biological functions (e.g., reproduction, foraging, and biotic interactions with other species) (Angilletta, 2009; Colinet Hervé et al., 2015). Since there are increasingly higher mean and extreme temperatures due to climate change, it is essential to understand the vulnerability of insects to this threat (Lovejoy & Hannah, 2005; Bellard et al., 2012). The threat is particularly dangerous in the tropics, where high baseline temperatures and small increases can severely impact insect metabolism and survival (Sunday et al., 2014). Moreover, phenotypic plasticity contributes very

little to heat tolerance in some insects, which need to migrate to higher elevations in response to increasing temperatures (García-Robledo et al., 2016), and heat tolerance evolves much slower than cold tolerance, leaving a lower potential for heat than cold adaptation (Bennett et al., 2021). Therefore, elevational gradients have served as important natural laboratories to evaluate plasticity and adaptation to different thermal regimes (García-Robledo et al., 2016; Pepin et al., 2022).

Bees have been used as a model group to understand the immediate responses to environmental changes, including thermal shifts (Bernier & Schoene, 2009). For instance, Pereboom and Biesmeijer (2003) found that small stingless bees get hotter faster than larger bees, which indicates that body size is a fundamental determinant of thermal tolerance. The honeybee *Apis mellifera* L. is of high economic and ecological importance worldwide, as it has a cosmopolitan distribution and pollinates a wide variety of native and cultivated plant species (Baena-Díaz et al., 2022; Cruz et al., 2022).



The ecological success of *A. mellifera* is linked to its ability to adapt to a wide range of climatic and environmental conditions (Dáttilo et al., 2022; Hodkinson, 2005; Obeso et al., 2018; Woyke et al., 2003). Recently, Sánchez-Echeverría et al. (2019) found that differences in thermal variability along small spatial scales of a few kilometers from the same city can influence the breadth of thermal tolerance of *A. mellifera*, indicating that the thermal tolerance of *A. mellifera* can be variable in sites with contrasting environmental characteristics such as urban and rural sites, in addition to demonstrating the plasticity and adaptation capacity of *A. mellifera* to different environmental conditions (Smith et al., 2015; Peters et al., 2016; Conrad et al., 2021; McCabe et al., 2022).

The morphological, behavioral, and physiological adaptations that *A. mellifera* possesses allow it to exist and develop in a great diversity of climatic conditions (Hodkinson, 2005; Obeso et al., 2018). In this sense, the climatic variability hypothesis states that as the range of climatic variation increases, the individuals adapted to these sites would have a broader range of thermal tolerance and, therefore, greater acclimatization capacities that allow them to cope with changes in weather conditions (Molina et al., 2012; Gutiérrez et al., 2016; Shah et al., 2021). Therefore, environmental conditions play a vital role not only in determining the presence of a species through short-term processes such as phenotypic plasticity (e.g., acclimatization) but also in long-term processes such as adaptive evolution by modifying its morphological and physiological characteristics (Chown & Nicolson, 2004; Angilletta, 2009; Hoiss et al., 2012). There is evidence of morphological and behavioral variation of *A. mellifera* in contrasting environments (Aizen et al., 2009; Parker et al., 2010). An interesting aspect is that the wide distribution of *A. mellifera* includes a wide range of elevations, inhabiting areas from 0 to 3,700 m.a.s.l. in both tropical and temperate zones (Cortina et al., 2019; Rabe et al., 2005; Dutton et al., 1981). According to the climate variability hypothesis, populations at higher elevations would present more significant climatic variability than those at low altitudes (Janzen, 1967). However, we do not know how environmental characteristics, body mass, and body size may differ among different populations along elevation gradients (Oyen et al., 2016; Peters et al., 2016).

Studies on the thermal tolerance of honeybees along elevational gradients have not been performed, even though these studies could help us to predict its response to future climatic change. Motivated by these concerns, the objective of this study was to determine the cold and heat tolerance, besides thermal range of *A. mellifera* workers at three different elevations, in addition to evaluating whether there is a relationship between body mass and thermal tolerance along an elevation gradient in the Mexican Transition Zone, an important biodiversity hotspot where the Nearctic and Neotropical biogeographic regions converge. At the population level, we expected the thermal tolerance and range to change along the elevation gradient, according to the climate variability

hypothesis, where the greatest range of temperature variation is found in sites with higher elevation. At the individual level, we postulated that larger-bodied workers could tolerate higher temperature extremes (both minimum and maximum) because they would gain and lose heat more slowly than smaller bees (i.e., they would not need to tolerate extreme temperatures) (Willmer & Unwin, 1981; Bishop & Armbruster, 1999).

Material and Methods

Sites studied

The study was conducted at three sites along an elevation gradient in the Mexican Transition Zone (Figure 1). Site A – “Centro de Investigaciones Costeras La Mancha” – is a coastal area located in the municipality of Actopan, Veracruz, Mexico (19°59'61.11" N and 96°38'36.11" W). The average annual temperature is 25 °C, with an elevation of 11 m.a.s.l., and annual rainfall varies between 1,200 and 1,500 mm. The driest season occurs between November and May; the rainy season begins in June, with the maximum precipitation peak in July (Castillo, 2006). The study area has various vegetation types, including coastal dunes, mangroves, low deciduous forests, medium sub-deciduous forests, and low floodable evergreen forests (Castillo & Medina, 2002). Site B – “Santuario del Bosque de Niebla” – is within a protected natural area of mountain cloud forest located in the city of Xalapa, Veracruz, Mexico (19°31'05" N - 96°56' 3" W) and presents an average elevation range of 1,324 m.a.s.l. This site has a temperate humid and semi-warm humid climate with an average annual temperature of 18 °C and an average annual rainfall of 1,500 mm (INEGI, 2016). Site C – “Comunidad de Miguel Hidalgo y Costilla” – is located in the municipality of Tlachichuca, Puebla, Mexico (19°04'56" N, 97°18'50" O), on the west face of the slopes of the Citlaltépetl volcano, at 3,304 m.a.s.l. with a predominance of coniferous forest and pine-oak forest vegetation, with average annual temperatures of 10 °C, as well as an average annual rainfall of 762 mm (Villegas et al., 2011).

Sampling honeybees

We collected *A. mellifera* worker bees between April and July 2022 between 08:00 and 16:00 hours (Figure 2). We caught individuals found foraging on flowers with the help of an entomological net and placed them inside plastic jars with sufficient ventilation. The individuals were transferred to the laboratory to weigh them and carry out the thermal tolerance experiments 4-6 hours after their capture.

Body mass and thermal tolerance

From each collected individual, we measured body mass and critical thermal limits. We placed captured bees in 1.5 mL Eppendorf tubes to measure fresh weight body mass with a digital scale before performing thermal tolerance

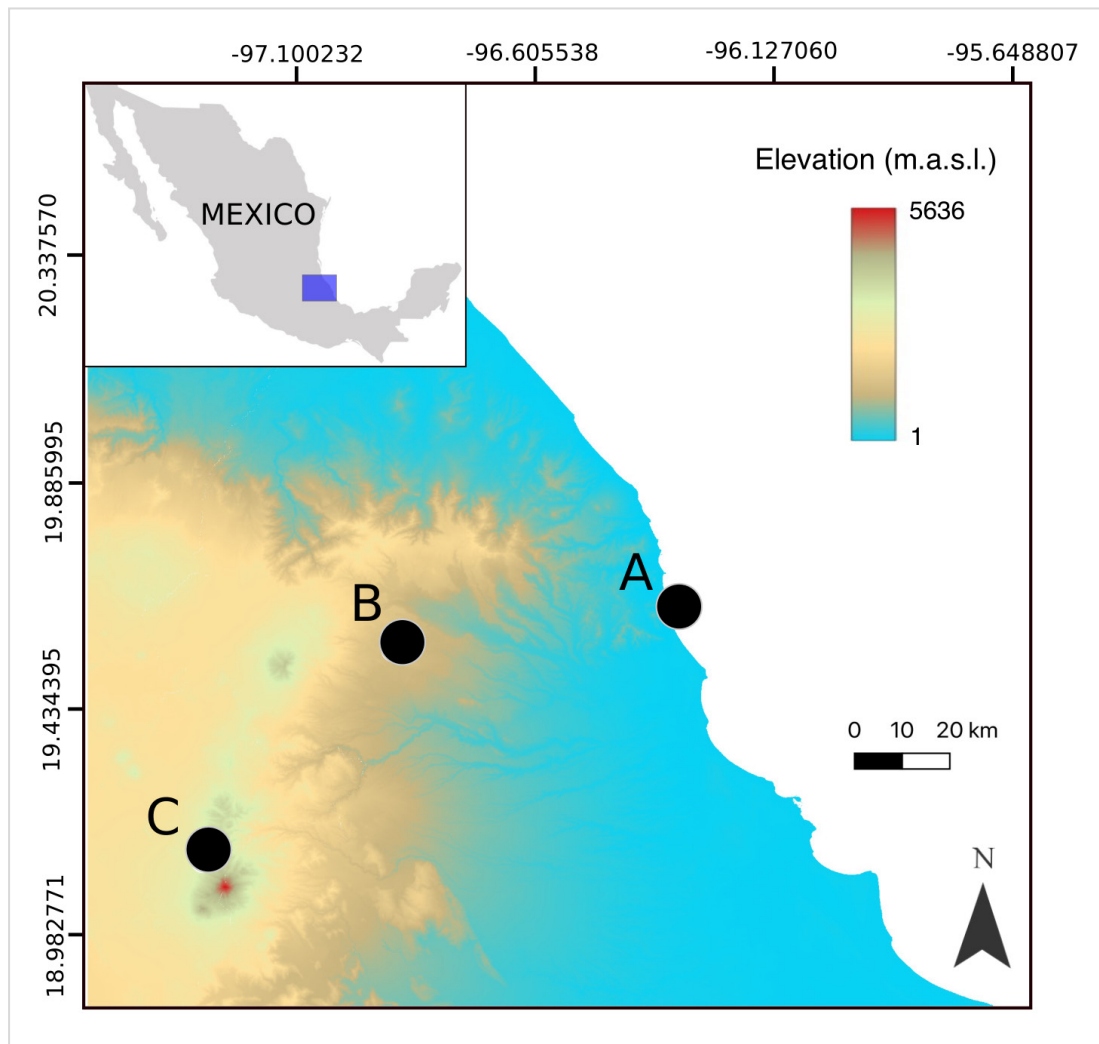


Fig 1. Geographical location of the three sites where *Apis mellifera* worker bees were collected. Site A – “Centro de Investigaciones Costeras La Mancha” (11 m.a.s.l.), Site B – “Santuario del Bosque de Niebla” (1,324 m.a.s.l.), and Site C – “Comunidad de Miguel Hidalgo y Costilla” (3,304 m.a.s.l.).

experiments (Profesional Digital Mini Scale, range 50g x 0.001g). As measurements of thermal tolerance, we used the critical thermal maximum and minimum (CTmax and CTmin), which are the high and low temperatures at which individuals lose muscular control. For this purpose, the tubes were placed in a Benchmark model BSH300 mini dry bath incubator. The initial temperature was 25 °C, which increased one degree per minute to measure CTmax and decreased the temperature one degree per minute for CTmin until reaching the critical temperature. A digital thermocouple (Kamtop) was placed inside the incubator (± 0.1 °C) to confirm the critical thermal values (Oyen et al., 2016; González et al., 2021). As some individuals do not recover from thermal tolerance measurements, different individuals were used to measuring CTmax or CTmin. We carried out the experiments with a total of 310 *A. mellifera* individuals, 114 from Site A (n = 60 for CTmax and n = 54 for CTmin), 88 from Site B (n = 27 for CTmax and n = 61 for CTmin), and 108 from Site C (n = 54 for CTmax and n = 54 for CTmin). In addition, we calculated

the range of thermal tolerance from the difference of the CTmax and CTmin values through bootstrapping, choosing 20 bee individuals from each of the two treatments to form 10 random pairs.

Data analysis

We used Generalized Linear Models (GLMs) with a Gaussian distribution to test whether the body mass, thermal tolerance (CTmax and CTmin), and range of thermal tolerance of *A. mellifera* (i.e., dependent variables) change among the three sampled elevations (i.e., independent variables). When significant differences were observed, we performed contrast analyses to determine differences between pairs of sites (Crawley, 2012). For residual and contrast analyses, we used the RT4Bio package (Reis-Jr, 2015). Moreover, we also used GLMs to test whether the body mass of *A. mellifera* workers was associated with their thermal tolerance (i.e., CTmax and CTmin) at each of the sampled sites. We used

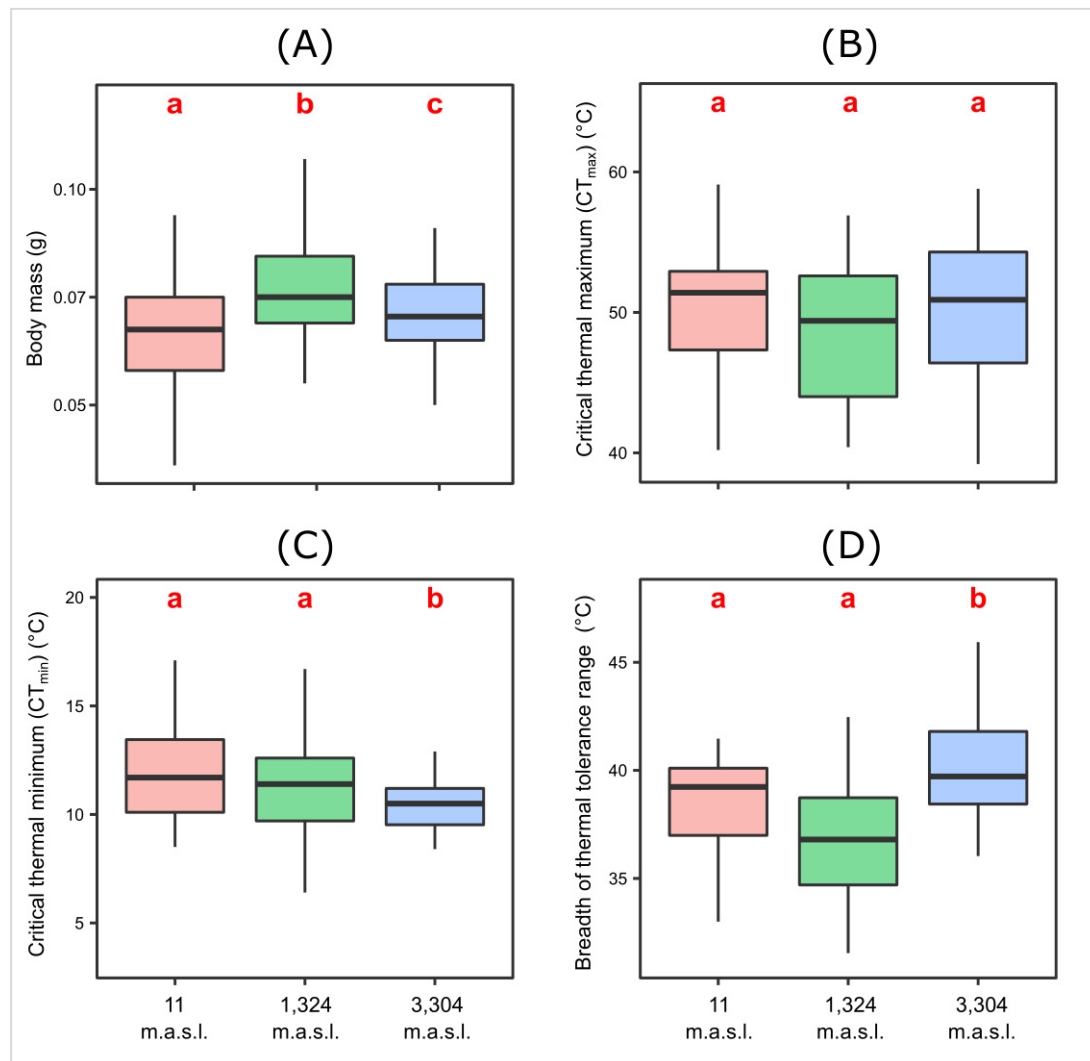


Fig 2. Values of (A) body mass (g), (B) critical thermal maximum (CT_{max}), (C) critical thermal minimum (CT_{min}), and (D) thermal tolerance range of *Apis mellifera* individuals at different elevations: Site A (11 m.a.s.l.), Site B (1,324 m.a.s.l.), and Site C (3,304 m.a.s.l.). Different letters above the bars denote significant differences between treatments ($P < 0.05$). At the same time, the median and the upper and lower quartiles of the values are represented in the box and whisker graphs.

χ^2 tests to estimate the goodness of fit of our models, which is the deviance divided by the dispersion parameter for the model. Analyses were done in software R, version 4.2.3 (R Development Core Team, 2023).

Results

We observed that the body mass of *A. mellifera* differed between the sampled elevations ($\chi^2 = 46.836$, $P < 0.001$) (Figure 2A). Specifically, *A. mellifera* individuals from the intermediate elevation (i.e., 1,324 m.a.s.l.) had higher body mass values (Mean \pm SD: 0.077 ± 0.0109 g) than individuals from the lower (0.066 ± 0.0123 g) and higher (0.071 ± 0.0096 g) elevation (i.e., 11 and 3,304 m.a.s.l., respectively). Regarding thermal tolerance, the thermal critical maximum (CT_{max}) of *A. mellifera* did not differ between the sampled sites ($\chi^2 = 3.267$, $P = 0.19$) (11 m.a.s.l.: 50.54 ± 4.74 °C;

1,324 m.a.s.l.: 48.46 ± 4.81 °C; 3,304 m.a.s.l.: 50.26 ± 5.29 °C) (Figure 2B). However, we observed that CT_{min} of *A. mellifera* differs between elevations ($\chi^2 = 13.691$, $P = 0.001$) (Figure 2C). In this case, individuals collected in the sites with lower and intermediate elevations had higher CT_{min} values than individuals at the higher elevation (11 m.a.s.l.: 11.90 ± 2.38 °C; 1,324 m.a.s.l.: 11.29 ± 2.64 °C; 3,304 m.a.s.l.: 10.35 ± 1.09 °C). We also observed that the thermal tolerance range in *A. mellifera* differed between elevations ($\chi^2 = 10.056$, $P = 0.009$) (Figure 2D). Specifically, we observed that *A. mellifera* collected at lower elevations had a lower thermal tolerance range (11 m.a.s.l.: 38.43 ± 3.85 °C; 1,324 m.a.s.l.: 37.01 ± 2.67 °C) compared to the individuals collected at the site with the highest elevation (3,304 m.a.s.l.: 39.71 ± 3.17 °C). Finally, we found no evidence that body mass was associated with thermal tolerance (i.e., CT_{max} and CT_{min}) at any of the three sampled elevations (All P-values > 0.05).

Discussion

Studies have shown that *Apis mellifera* can be present in sites where environmental conditions (e.g., temperature, humidity, precipitation, and wind speed) are highly contrasting (Maleszka, 2018). In this study, we found that different populations of *A. mellifera* have adapted to contrasting environmental conditions along an elevation gradient in the Mexican Transition Zone, a site within its non-native range. Specifically, we found differences in *A. mellifera* body mass, cold tolerance, and thermal range but no changes in heat tolerance at different elevations.

Previous studies have found that, in general, there is an increase in bee body size at higher elevations (Gerard et al., 2018; Noteen & Rehaan, 2020). These results confirm Bergmann's Rule (Bergmann, 1847), a fundamental rule of biogeography that postulates that there is a trend for the presence of larger individuals in colder habitats since larger animals would have a lower surface-area ratio, which would help reduce heat loss in colder environments. Similarly, we show that the body mass of *A. mellifera* populations was higher at higher elevation sites when compared to the lowest site located on the Gulf of Mexico coast. This finding is according to the reformulation of Bergmann's Rule made by Rensch (1938), which refers to populations living in cooler climates being generally larger than populations living in warmer regions. Therefore, the difference in body mass between *A. mellifera* populations between elevations could be related to different facets and selection mechanisms of these populations over acclimation time and environmental conditions since body size is a fundamental life-history trait that is directly related to the metabolism, fecundity, individual's movement, and dispersal capacity of the colonies (Blanckenhorn, 2000; Greenleaf et al., 2007; Al-Kahtani & Taha, 2021). Thus, it is unlikely that the size-thermal tolerance relationship results from evolutionary processes. Instead, it is more likely attributed to gene flow and adaptation to environmental conditions, considering the relatively short history of *A. mellifera* on the American continent, in contrast to its much longer evolutionary presence in Asia, Europe, and Africa (Harrison & Fewell, 2002; Han et al., 2012).

An important factor determining the spatial distribution of insects along elevational gradients is their thermal tolerance (Addo-Bediako et al., 2000; Overgaard et al., 2011). In this study, we observed that the CT_{max} of *A. mellifera* does not change along the elevation gradient. However, as expected, we observed that *A. mellifera* individuals captured at the highest elevation site had a lower CT_{min} (i.e., they tolerated colder temperatures) and a larger thermal tolerance range. The decrease in CT_{min} with increasing elevation has been found in other groups of insects, such as in ants (Bishop et al., 2017) and, recently, in bumblebees from the Andes (González et al., 2022), who explain this phenomenon by the rule de Brett (1959), which proposes that heat tolerance presents less

geographical variation than cold tolerance, as observed in this study. Furthermore, a recent study by Sánchez-Echevarría et al. (2019) showed that *A. mellifera* individuals collected in urban heat islands had higher CT_{min} values than those collected in rural sites, while CT_{max} did not differ. This information allows us to infer possible phenotypic plasticity of *A. mellifera* to environmental variation between sites and that thermal tolerance could be an adaptive response that should limit the distribution of *A. mellifera*. Therefore, identifying how and why *A. mellifera* is adaptively plastic to environments with contrasting temperatures, which can help us understand how honeybees adapt to environmental change and go through evolutionary dynamics (Fischman et al., 2017). Also, as expected, we found that *A. mellifera* populations from the highest elevation exhibited a greater thermal tolerance range, suggesting that high-elevation populations could be locally adapted to high temperature fluctuations. These results agree with the climate variability hypothesis, which states that climate becomes more variable at higher latitudes and elevations and that, therefore, species at these conditions should have a wider tolerance than species that are distributed at lower latitudes and elevations (Stevens, 1989; Chown et al., 2004). In addition, this result confirms the Janzen Rule that predicts a greater range of thermal tolerance where there is a more variable temperature environment, such as sites located at high elevations (Janzen, 1967; Gaston et al., 2009). It is essential to highlight that, to date, we do not know the processes of genetic divergence of *A. mellifera* in its non-native distribution despite being a species with a wide distribution. Future studies should analyze the local adaptation processes of *A. mellifera* to understand better how climate and temperature shape the distribution and ecological functions of this exotic and invasive species in ecosystems.

Many studies have shown that insect thermal tolerance is related to body size and may play a role in niche partitioning and biogeographical distribution patterns (Pereboom & Biesmeijer, 2003; Oyen et al., 2016). This is because larger organisms gain and lose heat more slowly than smaller species, which allows them to reach higher temperature excesses (Bishop & Armbruster, 1999). However, we did not find evidence of a relationship between body mass and thermal tolerance (CT_{max} or CT_{min}) in any of the studied elevations, despite body mass differing between elevations. These results are similar to those reported by Sánchez-Echevarría et al. (2019), where the authors also did not find a relationship between body mass and thermal tolerance (CT_{max} and CT_{min}) in *A. mellifera* from contrasting thermal environments. That is possible because thermal tolerance is not only limited by body size but also by other factors, such as the general physiological condition or the presence of additional environmental stressors (González-Tokman et al., 2021).

In this study, we found that different populations of *A. mellifera* differed in thermal tolerance along an elevation

gradient in the Mexican Transition Zone, with cold tolerance being more variable than heat tolerance, as in other systems (Bennett et al., 2021). Together, our findings indicate local adaptation or phenotypic plasticity in contrasting environments in the non-native distribution of *A. mellifera*, which would allow this species to expand its range of distribution. Studying thermal physiology along elevation gradients can help us to understand how the multiple factors related to global change could shape the thermal tolerance of the most important managed insect worldwide and its biotic interactions and ecosystem function. Finally, it is necessary to carry out future research to more clearly elucidate how the environment shapes *A. mellifera* populations at different spatial and temporal gradients.

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Authors' Contribution

JBB: Conceptualization, Methodology, Investigation, Formal analysis, Writing-Original Draft.

BR: Conceptualization, Methodology, Investigation, Formal analysis, Writing-Review & editing.

FB-D: Investigation, Writing-Review & Editing.

DG-T: Investigation, Resources, Writing-Review & editing.

WD: Supervision, Conceptualization, Methodology, Investigation, Writing-Review & editing, Project administration, Funding acquisition.

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