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Thermal limits of Africanized honey bees are influenced by temperature ramping rate but not by other experimental conditions

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ABSTRACT

Interest in assessing the critical thermal limits of bees is rapidly increasing, as these physiological traits are good predictors of bees' potential responses to extreme temperature changes, which is relevant in the context of global climate change. However, estimates of thermal limits may be influenced by several factors and published studies differ in experimental methods and conditions, such as the rate of temperature change (ramping rate) and feeding status, which might yield inaccurate predictions and limit comparisons across taxa and regions. Using Africanized honey bees as a model organism, we assessed the effect of ramping rate (0.25, 0.5, 0.75, 1.0 and 1.5 °C min^{-1}) and length of starvation (recently fed vs. fasted for 6, 12, and 18 h) on foragers' lower (CT_{Min}) and upper (CT_{Max}) thermal limits, as well as the effect of cold stress on CT_{Max}. In addition, we evaluated the two approaches currently used to assess CT_{Max} with a water bath: floating or submerging the testing vials in the bath. We found that critical thermal limits were influenced by ramping rates but not by the other assessed experimental conditions. On average, at ramping rates faster than 0.5 °C min^{-1}, bees displayed a CT_{Min} 1.1–2.6 °C lower and a CT_{Max} 5.3–6.9 °C higher than those of the slowest ramping rate. We discuss the implications of these results and provide suggestions for future thermal studies on bees.

Data accessibility statement

Authors agree upon archiving the data after the manuscript is accepted.

1. Introduction

Critical thermal limits, the minimum (CT_{Min}) and maximum (CT_{Max}) temperatures at which an animal can maintain muscle control, are key physiological traits for our understanding of an organism's ecology and evolution, as well as for predicting their responses to changes in land use and climate (Angilletta, 2009; Sunday et al., 2011; Hamblin et al., 2017; Nascimento et al., 2022). Such physiological traits are also commonly used in estimating thermal sensitivity indices, such as thermal safety margin and warming tolerance, which have been useful to make predictions of species vulnerability to climate change (Deutsch et al., 2008; Sunday et al., 2014; Clusella-Trullas et al., 2021). Thermal sensitivity indices are calculated as the difference between CT_{Max} and either an environmental (mean or maximum temperatures) or operative (optimal body temperature, field body temperature) temperature. The smaller the difference between these metrics of thermal tolerance and thermal environment, the more susceptible an organism is to global warming (Deutsch et al., 2008; Sunday et al., 2014; Clusella-Trullas et al., 2021). Because low estimates of CT_{Max} can be associated with low vulnerability whereas high estimates of CT_{Max} with high vulnerability, accurate estimates of critical thermal limits will result in better predictions of species' potential response to climate change. Thermal sensitivity indices are also prone to other limitations, such as the type of metric used to characterize the thermal environment (Clusella-Trullas et al., 2021). Nonetheless, critical thermal limits estimates are still an important part of the equation that can drastically alter the index value and thus influence the interpretation of a species' apparent susceptibility to climate change.

Critical thermal limits are measured under controlled conditions in the laboratory when organisms are exposed to either constant temperatures (static protocols) or to increasing or decreasing temperatures

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(dynamic protocols) (Lutterschmidt and Hutchison, 1997). However, these estimates may vary in response to a myriad of factors including life history traits (Baudier et al., 2018; Hamblin et al., 2017), abiotic conditions (Bujan et al., 2020; Roeder et al., 2021a), environmental stressors (González-Tokman et al., 2021; Gonzalez et al., 2022), and experimental conditions (Terblanche et al., 2007). For example, CT_{Max} can increase with increasing body size across species (Baudier et al., 2018; Oyen et al., 2016) while it may decrease with age and length of starvation (Nyamukondiwa and Terblanche, 2009; Chidawanyika et al., 2017). The rate of temperature change (ramping rate) in dynamic protocols is known to affect the average and variance of thermal limits' estimates (Terblanche et al., 2007; Chown et al., 2009; Oyen and Dillon, 2018), with slow changes in temperature resulting in either an increase or decrease of thermal limits (Terblanche et al., 2007). Food is also known to decrease CT_{Min} and increase CT_{Max} (Nyamukondiwa and Terblanche, 2009; Owen et al., 2013; Chidawanyika et al., 2017; Bujan and Kaspari, 2017).

Insects, as ectotherm organisms, are most vulnerable to climate change because of their limited capacity to regulate body temperature (Angilletta, 2009). Considering insects' diversity and keystone roles they perform within ecosystems, understanding their responses to climate change is one of the major challenges in climate change biology (Kellermann and van Heerwaarden, 2019). Among the diversity of interactions developed between insects and other organisms, pollination is an essential one for plant reproduction, ecosystem maintenance, and our food security (Klein et al., 2007). However, despite the ecological and economical importance of pollinating insects in ecosystems, aspects of their thermal biology remain unknown for most groups. For example, while critical thermal limits have been extensively explored in some insects, such as ants (Roeder et al., 2021a; Nascimento et al., 2022), those of bees and other pollinators remain relatively poorly studied.

To date, most studies assessing bees' critical thermal limits are from North America and are focused on understanding changes in bees' CT_{Max} in the context of landscape alteration, although some studies addressed its relationship with bees' invasiveness potential (da Silva et al., 2021) and foraging patterns (Gonzalez et al., 2020) (Table 1). A few other studies have also explored bees' thermal tolerance using different metrics, such as lethal thermal limits (Owen et al., 2013; Maia-Silva et al., 2021), supercooling point (Krunić and Stanisavljević, 2006; Scheffield, 2008; Owen et al., 2013), chill-coma recovery time (da Silva et al., 2021; Oyen et al., 2021), and time before heat stupor (Martinet et al., 2015; Zambra et al., 2020). Although the interest in the thermal biology of bees is rapidly increasing, studies differ in methodology, which likely affects estimates of critical thermal limits and thus potentially limit comparisons across taxa and regions, as well as predictions in response to climate change. For example, the rate of temperature change (ramping rate) in dynamic protocols vary widely among bee studies, from as slow as 0.1 °C min^{-1} (da Silva et al., 2021) to as fast as 1.5 °C min^{-1} (Gonzalez et al., 2020). Food is occasionally given to bees upon collection (as a sucrose solution) and prior to thermal assays (Gonzalez et al., 2020; da Silva et al., 2021). In addition, CT_{Min} and CT_{Max} are sometimes assessed on the same individual (Oyen et al., 2016; Maebe et al., 2021), first measuring CT_{Min} and then CT_{Max}, once bees have fully recovered from the cold. However, because prior exposure to low temperatures might affect CT_{Max} by increasing the duration of the experiment and exposure to potential confounding stressors, such as starvation and desiccation (Terblanche et al., 2011; Overgaard et al., 2012), other researchers have used different sets of individuals to assess each physiological trait (Sánchez-Echeverría et al., 2019).

The type of equipment used to assess bees' critical thermal limits is another major difference among studies, with some researchers using common water baths (Hamblin et al., 2017; da Silva et al., 2021; Gonzalez et al., 2020, 2022) while others employing more expensive, less accessible equipment, such as respirometers (Kovac et al., 2014) and environmental chambers (Burdine and McCluney, 2019; Maebe et al., 2021). Generally, researchers leave the testing vials floating in the water bath, rotating them regularly to equalize the temperature throughout the vial (García-Robledo et al., 2016, 2018). However, other researchers submerged the vials in the water bath (Boyle et al., 2021; Gonzalez et al., 2020, 2022) to maintain an equilibrium between the temperature of the water and the vial throughout the experiment. Both approaches have been reported in thermal studies with bees, but no study has validated

Table 1
Summary of studies assessing the critical thermal minima (CT_{Min}) and maxima (CT_{Max}) of bees. Abbreviations: T_0 = initial temperature during assays; AT = acclimation time; TRR = temperature ramping rate; F = feeding – = No; + = Yes; $ = tested on the same individual; ? = data unknown.

Location	T_0 (°C)/ AT (min)	TRR (°C min^{-1})	F	Assessed thermal limit	Equipment	Taxon	Topic	Reference
Austria/Italy	25/?	0.25	–	CT_{Max}	Respirometry chamber	*Apis mellifera*	Subspecies comparisons	Kovac et al. (2014)
Wyoming, USA	22/10	0.25/ 0.50	–	CT_{Min}/ $CT^{\$}_{Max}$	Aluminum block	*Bombus huntii, B. bifarius, B. sylvicola*	Elevation	Oyen et al. (2016)
Michigan, USA	22/10	0.1, 0.25, 1.0	+	CT_{Min}/ $CT^{\$}_{Max}$	Aluminum block	*Bombus impatiens*	Acclimation, age, feeding	Oyen and Dillon (2018)
Ohio, USA	25/?	0.50	+	CT_{Max}	Environmental chamber	*A. mellifera, B. impatiens, Agapostemon sericeus*	Urban vs. rural	Burdine and McCluney (2019)
North Carolina, USA	25/20	0.50	–	CT_{Max}	Water bath/partially submerged	*Agapostemon virescens, Bombus bimaculatus, B. griseocollis, B. impatiens, Ceratina calcarata, C. strenua, Halictus ligatus/poeyi, Lasioglossum imitatum, L. bruneri, Megachile campanulae, M. exilis, M. mendica, M. rotundata, Ptilothrix bombiformis, Xylocopa virginica*	Urban vs. rural	Hamblin et al. (2017)
Hidalgo, Mexico	25/≥60	0.50	+	CT_{Min}/ CT_{Max}	Refrigerated/ heating circulating water bath	*A. mellifera*	Urban vs. rural/ acclimation	Sánchez-Echeverría et al. (2019)
Lesbos, Greece	26/15	1.5	+	CT_{Max}	Water bath/ submerged	*Xylocopa violacea, X. olivieri, X. iris*	Diurnal vs. nocturnal/ elevation	Gonzalez et al. (2020)
California, USA	25/15	0.25	+	CT_{Min}/ CT_{Max}	Aluminum block	*B. vosnesenskii*	Gene expression	Pimsler et al. (2020)
Fiji	25/?	0.1	+	CT_{Max}	Water bath/floating	*Homalictus fijiensis, Braunsapis puangensis, Ceratina dentipes*	Invasive vs. native species	Da Silva et al. (2021)
Belgium	10,20/ 30	0.5/ 0.35	+	CT_{Min}/ $CT^{\$}_{Max}$	Environmental chamber	*B. terrestris*	Subspecies comparisons	Maebe et al. (2021)

these methods.

As an attempt to begin addressing these methodological challenges in bee thermal studies, and to generate discussion for more suitable and standard protocols, here we used Africanized honey bees (*Apis mellifera* L.) as a model organism to answer the following questions: how does the temperature ramping rate and food influence estimates of CT_{Min} and CT_{Max}? Does exposure to low temperatures, during measurement of CT_{Min}, influence CT_{Max}? Does CT_{Max} vary between bees tested inside floating and submerged glass vials in a water bath?

2. Materials and methods

2.1. Study site and experiments

We conducted experiments between February and May of 2021 with Africanized honey bee foragers from two experimental Langstroth hives in Tenjo, Cundinamarca, Colombia (4°51.410′N, 74°06.468′W, 2589 m, daily average 5.5–28.4 °C, 42.4–96.4% RH). Both colonies from these hives were captured locally from feral swarms, a common practice used by Colombian beekeepers that has contributed to maintain a high level of Africanization in the country (Tibatá et al., 2018). Bees inhabited the hives for at least six months prior to our experiments. We chose the Africanized honey bee as a model organism for this study because it is among the few bee species that are locally abundant and readily accessible at these high elevations in the Andes. We trained bees from each hive to forage at a feeder containing a 50% sucrose solution scented with either lavender or mint. We captured bees at the feeder between 9:00 and 11:00 h with a plastic vial, which we then capped with fabric (1 mm mesh). Unless otherwise indicated, we fed bees ad libitum prior to assays and tested them within 30 min of collection. To answer our questions, we assessed either CT_{Max} or both CT_{Min} and CT_{Max} in the following four experiments, each of which controlled for one or more independent variables:

Experiment 1. To assess the effect of temperature ramping rates on thermal limits, we measured bees' CT_{Min} and CT_{Max} from assays using ramping rates of 0.25, 0.5, 0.75, 1.0, and 1.5 °C min^{-1}. We measured CT_{Min} and CT_{Max} on the same individual and used a different set of bees for each ramping rate. Because a slow assay allows more time for acclimation than a fast one, we therefore expect CT_{Min} to decrease and CT_{Max} to increase with slower ramping rates.

Experiment 2. To assess the effect of feeding on bees' thermal limits, we fed bees to satiation with a 50% sucrose solution using a micropipette. Then, we measured CT_{Min} and CT_{Max} on recently fed bees and bees fasted for 6, 12, and 18 h. We chose to feed bees to satiation at the beginning of this protocol because pilot assays keeping bees for more than 6 h without feeding after collection at the feeders resulted in high mortality. During the experiment, we kept bees at room temperature inside of a cardboard box containing a water-filled open container to maintain humidity (16.6–20.8 °C, 65.6–80.0% RH). We measured CT_{Min} and CT_{Max} on the same individual and randomly chose a different set of bees for each fasting period. We chose an intermediate ramping rate of 0.5 °C min^{-1} to reduce assay time and potential confounding physiological stressors, such as dehydration. Because food is known to decrease CT_{Min} and increase CT_{Max} (Nyamukondiwa and Terblanche, 2009; Chidawanyika et al., 2017), we expect CT_{Min} to increase and CT_{Max} to decrease with increasing fasting period.

Experiment 3. To assess whether exposure to low temperatures, as when measuring CT_{Min} could affect CT_{Max}, we compared CT_{Max} between bees in which we measured CT_{Min} first (cold exposed) and those in which we only measured CT_{Max}. As in experiment 2, we used a ramp rate of 0.5 °C min^{-1}. If measuring thermal limits on the same individual leads to confounding effects, such as cellular damage (Overgaard et al., 2012), we expect CT_{Max} to decrease in cold-exposed individuals.

Experiment 4. We compared CT_{Max} between bees tested inside sealed glass vials either floating or submerged in a water bath. We increased the water bath temperature 1 °C every 2.5 min to attain an equilibrium between water and vial temperature. We expect similar estimates of CT_{Max} between these designs because bees inside vials will reach their upper thermal limit independently of whether vials are actively rotated or passively warmed when they are submerged in the water bath.

2.2. Measurements of thermal limits

For experiments 1–3, we measured CT_{Min} and CT_{Max} using the Elara 2.0 (IoTherm, Laramie, WY), a portable fully programmable heating/cooling anodized aluminum stage designed for precision temperature control of laboratory and field samples. The stage was modified with a Styrofoam cooler and clear acrylic lid to minimize the impact of airflow across the aluminum sample stage and maintain temperature stability across all vials. We placed bees individually inside glass vials (12 × 35 mm, 1.85 cm^3) and plugged them with a moistened cotton ball (~0.2 mL of distilled water per cotton ball) to ensure enough humidity during the assays. We used an initial temperature of 22 °C and held bees for 10 min at this temperature before increasing it or decreasing it at the rate indicated for each experiment. We placed vials horizontally on the stage to avoid bees from climbing along the vial. To estimate the temperature inside the vials, we placed a K-type thermocouple inside two empty glass vials plugged with a cotton ball. We individually tracked these vial temperatures using a TC-08 thermocouple data logger (Pico Technology, Tyler, TX, USA). Thus, we report the temperatures inside tubes not the temperatures displayed by the thermostat of the aluminum stage. As an approximation of bees' thermal limits, we used the temperature at which bees show signs of curling (CT_{Min}, Oyen and Dillon, 2018) or lost muscular control, spontaneously flipping over onto their dorsa and spasming (CT_{Max}, Lutterschmidt and Hutchison, 1997; García-Robledo et al., 2016, 2018).

For experiment 4, we used a water bath with a volume of 2L controlled by a thermostat (5–90 °C; ThermoFisher Scientific™ Precision™ GP02, accuracy of ±0.1 °C). To measure CT_{Max}, we placed bees individually in screw cap glass vials (17 × 60 mm, 7.4 cm^3) sealed with parafilm. Depending on the assay, we either left vials floating on the water bath or submerged them horizontally approximately 1 cm by attaching them to a metal tray. We used an initial temperature of 22 °C and held bees for 10 min at this temperature before increasing it 1 °C every 2.5 min. To estimate the temperature inside the tubes, we placed an iButton data logger (weight: 3.104 g; DS1923 Hygrochron™; Maxim Integrated, San Jose, California) inside a glass vial, which, depending on the assay, we either submerged or left floating on the water bath. Thus, we report the temperatures inside tubes not the temperatures displayed by the thermostat of the water bath. As in studies with ants (Baudier et al., 2015, 2018), prior experiments indicated that bees held in similar sealed glass vials adjacent to the water bath at room temperature survived through the duration of the assays.

2.3. Data analyses

We conducted statistical analyses in R version 4.0.3 (R Core Team, 2018) and created boxplots using GraphPad Prism version 7.04 (GraphPad Software, San Diego, CA, USA). We implemented a linear mixed-effect model (LMM) using the lmer function in the lme4 package version 1.1–30 (Bates et al., 2015). We used either CT_{Min} or CT_{Max} as a response variable in all models. For experiment 1, we used ramping rate (0.25, 0.5, 0.75, 1.0 and 1.5 °C min^{-1}) as a fixed factor. For experiment 2, we used time interval (0, 6, 12, and 18 h) as a fixed factor. For experiments 3 and 4, we used exposure to cold and testing condition (floating vs. submerged) as fixed factors. We used colony identity as a random factor in all models. Treating colony identity as a fixed factor in a linear model yielded similar results (Table S1). We assessed the significance of fixed effects using a Type II Wald χ^2 test with the car package version 3.1–0 (Fox and Weisberg, 2011). When factors and

factor interactions were significant, we used the lsmeans package version 2.30–0 (Lenth, 2016) to conduct multiple pairwise comparisons with Bonferroni adjustment to assess for differences among groups. We compared variance in thermal tolerance using *F*-tests with the var. test function and Levene's tests with leveneTest function from the car package.

3. Results

The critical thermal minima and maxima of Africanized honey bee foragers varied among assays using different temperature ramping rates (CT_{Min}, Wald $\chi^2 = 120.97$; CT_{Max}, $\chi^2 = 196.42$, $P < 0.001$ and $DF = 4$ in both cases; Table S2). In general, CT_{Min} tended to decrease with increasing ramping rate whereas CT_{Max} tended to increase (Fig. 1a). Pairwise comparisons with Bonferroni adjustment (Table S3) indicated differences between the CT_{Min} of bees exposed to the slowest ramping rate (0.25 °C min^{-1}) and that of remaining rates, as well as between 0.5 and 1.0 and 1.5, and between 0.75 and 1.5 °C min^{-1}. The CT_{Min} of bees in ramping rates of or faster than 0.5 °C min^{-1} was, on average, between 1.1 and 2.6 °C lower than that displayed by bees in the slowest ramping rate. Variance was similar among assays (Levene's test: $F_{(4, 222)} = 1.94$, $P = 0.10$). We observed a similar pattern for CT_{Max} (Fig. 1b, Tables S2 and S3), with bees in ramping rates of or faster than 0.5 °C min^{-1} displaying an average CT_{Max} of 5.3–6.9 °C higher than that of bees exposed to the slowest ramping rate. However, variance was significantly different among assays ($F_{(4, 225)} = 31.29$, $P < 0.001$), being between two and three times greater at the slowest ramping rate than in other ramping rates (Table S2).

In contrast, CT_{Min} and CT_{Max} of Africanized honey bee foragers did not change after they were starved for up to 18 h (CT_{Min}, $\chi^2 = 4.585$, $P = 0.205$; CT_{Max}, $\chi^2 = 2.561$, $P = 0.464$, $DF = 3$ in both cases, Fig. 1c and d, Table S4). However, variance increased (44–50%) with the length of starvation, especially in CT_{Min} assays (CT_{Min}, $F_{(3, 80)} = 3.50$, $P = 0.02$; CT_{Max}, $F_{(3, 80)} = 2.15$, $P = 0.10$). Exposing bees to low temperatures to measure their CT_{Min} prior to assessing CT_{Max}, did not influence the latter average value ($\chi^2 = 2.154$, $DF = 1$, $P = 0.142$; Fig. 1e, Table S5). However, variance in CT_{Max} was greater (~29%) when measured independently ($F_{(33, 34)} = 0.50$, $P = 0.05$). Finally, we observed a similar CT_{Max} average value between bees tested in sealed vials floating on the water bath and those submerged ($\chi^2 = 0.975$, $DF = 1$, $P = 0.324$; Fig. 1f, Table S5). Variance in CT_{Max} was similar between these assays (CT_{Max}, $F_{(30, 27)} = 1.30$, $P = 0.50$).

4. Discussion

Estimates of the average critical thermal minima and maxima of Africanized honey bee foragers were significantly influenced by temperature ramping rate but not by the length of starvation, prior cold

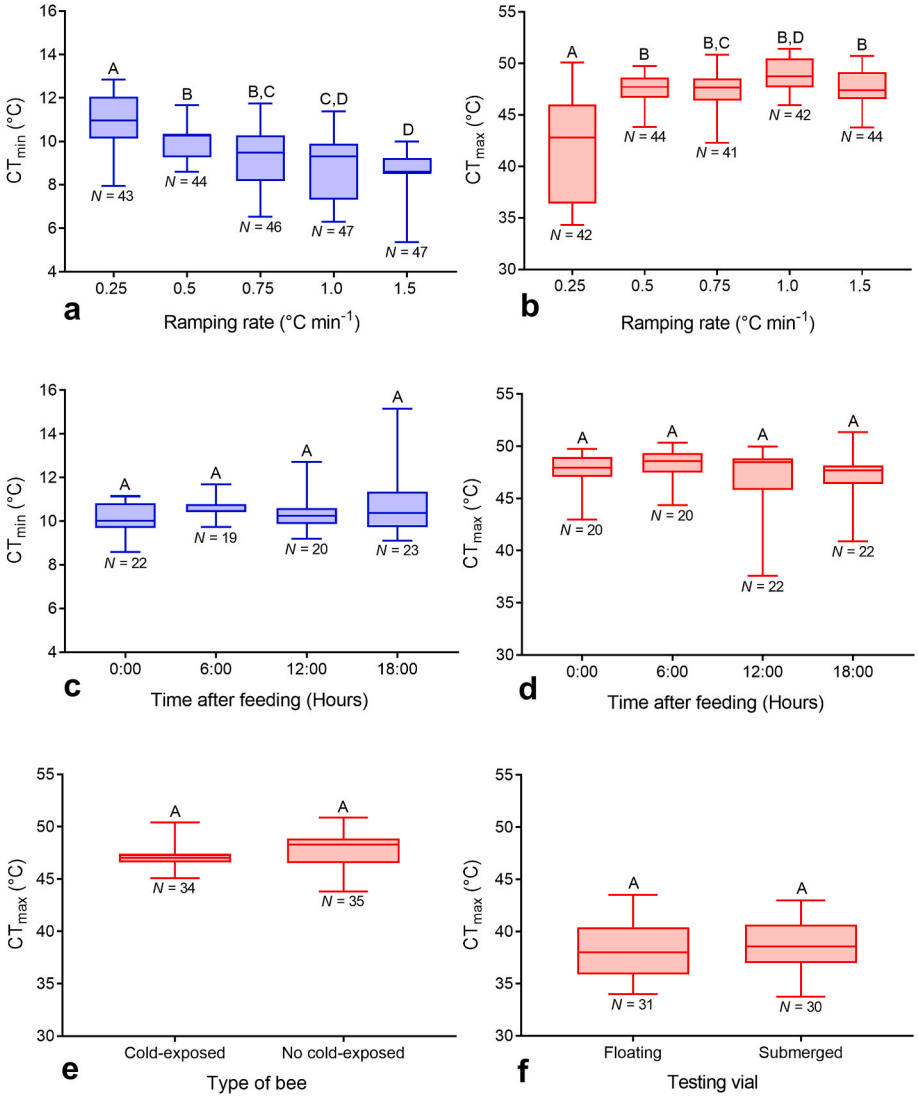

Fig. 1. Boxplots showing critical thermal minima (CT_{Min}) and maxima (CT_{Max}) of Africanized honey bees in the eastern Andes of Colombia. **a, b,** comparison of CT_{Min} and CT_{Max} among ramping temperature rates. **c, d,** comparison of CT_{Min} and CT_{Max} at different lengths of starvation. **e,** comparison of CT_{Max} between cold-exposed bees during measurement of CT_{Min} and no cold-exposed bees. **f,** comparison of CT_{Max} between bees tested in floating and submerged vials in the water bath. Boxplots display median, quartiles, and extreme values. For each figure, different letters above boxplots represent significant differences ($P < 0.05$).

exposure, or vial placement in water bath trials. CT_{Min} was significantly lower (1.1–2.6 °C) and CT_{Max} was significantly higher (5.3–6.9 °C) at ramping rates of or faster than 0.5 °C min^{-1} when compared to those displayed by bees at 0.25 °C min^{-1}, the slowest ramping rate we used (Fig. 1a and b). The impact of ramping rate on thermal tolerance has been subject of inquiry for a long time (e.g., Lutterschmidt and Hutchison, 1997; Mitchell and Hoffmann, 2010; Overgaard et al., 2011, 2012; Nguyen et al., 2014), with studies demonstrating differential responses among species and traits, ranging from an increase or decrease in one or both thermal limits to no response (e.g., Terblanche et al., 2007; Chown et al., 2009; Oyen and Dillon, 2018; Kovacevic et al., 2019). Several explanations might underly the impact of ramping rate on estimates of critical thermal limits, including variability in experimental methods, the precision of thermal ramping equipment, as well as physiological differences related to the duration of exposure to stressful conditions (Terblanche et al., 2007; Jørgensen et al., 2021).

Oyen and Dillon (2018) observed a similar improved response in the critical thermal limits of the common eastern bumble bee, *Bombus impatiens* Cresson, where CT_{Min} decreased and CT_{Max} increased in response to faster ramping rates. These authors suggested that ramping rate influences the exposure time to physiologically stressful temperatures because bees' thoracic temperatures were similar between individuals exposed to a slow (0.1 °C min^{-1}) and a fast (1.0 °C min^{-1}) ramping rate. Another possible explanation for this pattern among bees is desiccation, which is known to decrease thermal tolerance in some insects (Nguyen et al., 2017), although the opposite effect has also been documented (Edney, 1977; Mutamiswa et al., 2021). Bees appear to be sensitive to desiccation (Burdine and McCluney, 2019) and slow heating or cooling rates might shift the partial pressure deficit of water within experimental containers, thus altering the drying power of the air. Future studies should address the effect on desiccation on bees' thermal tolerance.

Although our results fall within the range of variation in the responses documented for species to date (Terblanche et al., 2007; Chown et al., 2009), they are not consistent with our initial expectation that a slow ramping rate would decrease CT_{Min} and increase in CT_{Max} because it would allow more time for acclimation than a fast one. While ramping rate influenced the average estimates of CT_{Min} and CT_{Max}, it only affected the variance of CT_{Max}. The effect of ramping rate on the variance of thermal limits' estimates, which is relevant for accurate estimates of heritable variation of the trait itself, also vary significantly among species (Chown et al., 2009). Our results are like those reported for workers of the Argentine ant, *Linepithema humile* (Mayr), as variance in our CT_{Max} experiments was largest at the slowest ramping rate (Chown et al., 2009). However, Argentine ants also displayed the largest variance in CT_{Min} at the slowest ramping rate while in our experiments variance was similar among CT_{Min} assays.

The best ramping rate to use in dynamic protocols has been the subject of debate, with some favoring slow ramping rates that resemble the slow temperature changes of the environment while others favoring fast ramping rates that reduce the impacts of concurrently occurring physiological stressors, such as starvation and dehydration (Terblanche et al., 2007; Kingsolver and Umbanhowar, 2018). However, independent of the preferred ramping temperature, our results suggest that the values of bees' thermal limits obtained in the few studies with slow ramping rates are not directly comparable with those obtained using medium to fast ramping rates (Table 1). Although the study of Gonzalez et al. (2020) used the fastest ramping rate (1.5 °C min^{-1}) recorded among bees to measure CT_{Max}, their data might still be comparable with other studies using 0.5 °C min^{-1}, as we observed a stronger response in CT_{Min} between 0.5 and 1.5 °C min^{-1} than in CT_{Max} (Fig. 1a and b). Because the relationship between environmental and core body temperatures is consistent across a broad range of ramping rates (Oyen and Dillon, 2018), even in large heterothermic bees, an intermediate ramping rate such as 0.5 °C min^{-1} reduces the time required for each experiment and minimizes the effect of confounding physiological stressors such as dehydration or starvation. We are aware that access to equipment is a major limiting factor, particularly in tropical, developing countries. However, García-Robledo et al. (2020) described portable, inexpensive, and easily accessible devices to measure insects' thermal limits in the field that could potentially alleviate this limitation.

Ingestion of carbohydrates can significantly increase CT_{Max} in insects. For example, workers of the canopy ant *Azteca chartifex* fed with a 10% sucrose solution displayed a CT_{Max} 5 °C higher than fasted individuals (Bujan and Kaspari, 2017). One mechanism to explain this response is that sucrose can be stored as glycogen, which can then be used to generate ATP (adenosine triphosphate) in the synthesis of heat shock proteins (Suarez et al., 1996; Bujan and Kaspari, 2017). Our results indicate that this is not the case for Africanized honey bees, which agree with previous observations on *B. impatiens* by Oyen and Dillon (2018) who compared CT_{Min} and CT_{Max} between fed and unfed individuals. Although we did not observe significant differences in the average values of CT_{Min} and CT_{Max}, variance increased (44–50%) with the length of starvation. Body mass could explain some of this variability, with heavier bees being more resistant to starvation than smaller, lighter bees (Oyen et al., 2016), which is exacerbated as time since feeding increases. Age could be another source of variation, with younger bees being more resistant to starvation than older bees (Oyen and Dillon, 2018). However, we were unable to measure body mass or to control for age, as we collected bees from a feeder. Future studies should address these issues.

Measuring CT_{Min} and CT_{Max} on the same individual could lead to confounding effects due to the production of stress proteins or the cumulative impacts of cellular damage at stressful temperatures (Overgaard et al., 2012). This does not seem to be the case for bees. As previously documented for *B. impatiens* (Oyen and Dillon, 2018), we did not observe an effect of exposure to cold on average estimates of CT_{Max}, as when bees are exposed to low temperatures during CT_{Min} assays. However, and unlike *B. impatiens* that displayed a similar variance, honey bees displayed a greater variance in the CT_{Max} when measured independently than when measured after the CT_{Min} assays on the same individual. Thus, while exposure to low temperatures might not influence the average estimate of CT_{Max}, it might affect its variance. Further studies need to assess other bee species to determine if such differential responses are species specific. From a practical point of view, these results are relevant because most bee species are solitary and many are rather rare, so measuring both physiological traits on the same individual is almost a necessity.

We found that submerging the vials did not affect the average or variance of CT_{Max} (Fig. 1f.). Thus, submerging the vials is unnecessary and it also increases preparation time, as these must be sealed with parafilm and attached to a tray, which also reduces the available space and the number of bees that can be simultaneously tested. Although we did not intend to assess differences between methods (water bath vs. aluminum stage), we observed that estimates of CT_{Max} from the water bath were much lower than those obtained on the aluminum stage (38 °C vs. > 42 °C), even lower than those from the slowest ramping rate (Fig. 1f; Tables S2, S4, S5). Gonzalez et al. (2022) reported similar low average values of CT_{Max} for the European honey bee in Kansas, which were obtained submerging the vials in the water bath as described in this work. Pilot experiments on the same bee population also show a CT_{Max} 6 °C higher when estimated on the aluminum stage, which is comparable with other estimates for honey bees using respirometry (Kovac et al., 2014), environmental chamber (Burdine and McCluney, 2019), or more sophisticated equipment (Sánchez-Echeverría et al., 2019).

Because temperature increases metabolic rate and oxygen consumption, bees inside warmed sealed vials are likely to experience hypoxia. Oxygen limitation is known to reduce CT_{Max} in marine and terrestrial organisms, although its effects on insects is mixed, with some species being unaffected by hypoxic conditions (e.g., Klok et al., 2004; Javal et al., 2019). These differences in CT_{Max} between methods exemplify the potential limitations when attempting to compare estimates

between studies using water baths and studies using digital dry blocks, or between studies that have the same average ramp rate but with different increments. Other methodological aspects known to affect thermal limits estimates, such as acclimation time and starting temperature of thermal assays (Terblanche et al., 2007), also vary among bee studies. For example, acclimation time ranges from 10 to more than 60 min while starting temperature from 10 to 26 °C (Table 1). Thus, further thermal bee studies should address the potential effects of these variables.

Hamblin et al. (2017) used an interesting set up to avoid hypoxic conditions inside the vials. These authors used plaster of Paris in the bottom of glass vials to submerge them partway in the water bath but closed them with a cotton ball above the water level (Youngsteadt, E., personal communication). Oxygen limitation might not be a problem with this design, but Hamblin et al. (2017) did not assess the CT_{Max} of honey bees, so it is unclear whether their CT_{Max} estimates were similarly low to our water bath experiment or improved by the change in methodology. The vertical orientation of the vials within the water bath might be a limitation of this design, as it encourages bees to move up due to their negative geotaxis or to seek a thermal refuge at the top of the vial. This can be avoided by coating the inside of the vials with Insect-a-slip or Fluon® (Oyen and Dillon, 2018), but this commercial product might not be readily available. In addition, preparing the vials might add extra work and time, particularly if experiments are conducted in remote locations in the field.

Although not conceptually new, our study is the first to investigate these aspects of the thermal biology of honey bees, the single most valuable managed pollinator in the world (Hung et al., 2018). It is also the first to highlight the methodological variability in todays' bee thermal research and the potential limitations when attempting to compare estimates of thermal limits among studies. Thus, our study provides the basis for methodological improvement and consistency of future thermal studies on bees, a relevant practical implication given the increasing interest in bees' thermal research because of the expected negative effects of climate change on pollinators and pollination (Kerr et al., 2015; Walters et al., 2022). Estimates of bees' thermal limits will have a profound effect on estimates of their thermal sensitivity to extreme temperature changes as well as on predictions of global warming impacts. For example, if thermal sensitivity indices are calculated with the low estimates of CT_{Max} obtained using either the slowest ramping rate on the aluminum stage or the water bath, these will result in index values that underestimates the vulnerability of honey bees to climate change. Broad scale comparisons using these low estimates of CT_{Max} will erroneously indicate low heat tolerance in Andean populations of honey bees. Thus, accurate thermal limits estimates will allow us to make better predictions of their changes in population, community (Hamblin et al., 2017), and species composition (Roeder et al., 2021b).

Although our results overall agree with previous observations on a single North American bumble bee species (Oyen and Dillon, 2018), we must be cautious in assuming that other species of bees might also exhibit thermal limits that are not influenced by the same experimental conditions. First, honey bees and bumble bees are phylogenetically related as they belong to the corbiculate clade (Michener, 2007). Second, they are eusocial species, which represent the minority of bees among the more than 20,000 species worldwide, and they can thermoregulate their nests. Thus, their similar responses could be due to their phylogenetic relationship or shared life history traits. At least for bumble bees, critical limits are potentially driven by genetic mechanisms and tied to aspects of local climate (Pimsler et al., 2020). Studies on ants have shown that CT_{Min} is influenced by microclimate while CT_{Max} is more phylogenetically constrained (Bujan et al., 2020; Leahy et al., 2022). Further studies will reveal whether these patterns are consistent among other hymenopterans. Undoubtedly, we need a comparative study that assesses bees' responses to experimental conditions using a wide range of taxa.

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Authorship statement

Victor H. Gonzalez: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing-Original Draft, Writing-Review & Editing, Project administration, Funding acquisition. Kennan Oyen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing-Original Draft, Writing-Review & Editing, Funding acquisition. Omar Ávila: Methodology, Validation, Investigation, Resources, Writing-Review & Editing. Rodulfo Ospina: Methodology, Validation, Investigation, Resources, Writing-Review & Editing.

Declaration of competing interest

The authors of this study have no conflicts of interest to declare.

Data availability

All relevant data are within the paper and its supporting information files.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2022.103369.

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