*Supplementary Files*

# Low heat tolerance and high desiccation resistance in nocturnal bees and the implications for nocturnal pollination under climate change

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Running title: Thermal and desiccation tolerance of nocturnal bees

**Table 1S.** Results of pairwise comparisons with Bonferroni adjustment of the critical thermal minima ($CT_{Min}$) and maxima ($CT_{Max}$) among focal species. Only significant ($P < 0.05$) comparisons of the 136 total tests for each thermal trait are listed.

| **Comparison** | **$CT_{Min}$ *P*-value** | **Comparison** | **$CT_{Max}$ *P*-value** |
|---|---|---|---|
| *M. amoena* vs *E. bombiformis* | 0.009 | *M. amoena* vs *E. bursigera* | 0.010 |
| *M. amonea* vs *T. ziegleri* | <0.0001 | *M. genalis* vs *E. bursigera* | 0.001 |
| *M. amonea* vs *T. angustula* | <0.0001 | *E. pulchra* vs *X. aeneipennis* | 0.013 |
| *M. amonea* vs *T. fuscipennis* | <0.0001 | *E. bursigera* vs *E. imperialis* | 0.016 |
| *M. amonea* vs *X. aeneipennis* | <0.0001 | *E. imperialis* vs *X. aeneipennis* | 0.014 |
| *M. genalis* vs *E. crassipunctata* | 0.0001 | | |
| *M. genalis* vs *E. bombiformis* | 0.01 | | |
| *M. genalis* vs *T. ziegleri* | <0.0001 | | |
| *M. genalis* vs *T. angustula* | <0.0001 | | |
| *M. genalis* vs *T. fuscipennis* | <0.0001 | | |
| *M. genalis* vs *X. aeneipennis* | <0.0001 | | |
| *C. currani* vs *E. bombiformis* | 0.003 | | |
| *C. currani* vs *T. angustula* | 0.036 | | |
| *C. currani* vs *X. aeneipennis* | < 0.0001 | | |
| *E. pulchra* vs *C. crassipunctata* | 0.012 | | |
| *E. pulchra* vs *E. tridentata* | 0.011 | | |
| *E. pulchra* vs *E. bombiformis* | 0.003 | | |
| *E. pulchra* vs *T. ziegleri* | 0.023 | | |
| *E. pulchra* vs *T. angustula* | 0.016 | | |
| *E. pulchra* vs *T. fuscipennis* | 0.012 | | |
| *E. pulchra* vs *X. aeneipennis* | <0.0001 | | |
| *E. bursigera* vs *T. ziegleri* | 0.006 | | |
| *E. bursigera* vs *T. angustula* | 0.002 | | |
| *E. bursigera* vs *T. fuscipennis* | 0.004 | | |
| *E. bursigera* vs *X. aeneipennis* | <0.0001 | | |
| *E. crassipunctata* vs *E. bombiformis* | 0.0001 | | |
| *E. crassipunctata* vs *E. frontalis* | 0.012 | | |
| *E. crassipunctata* vs *X. aeneipennis* | <0.0001 | | |
| *E. cybelia* vs *E. bombiformis* | 0.0001 | | |
| *E. cybelia* vs *X. aeneipennis* | <0.0001 | | |
| *E. imperialis vs E. bombiformis* | 0.0001 | | |
| *E. imperialis* vs *X. aeneipennis* | <0.0001 | | |
| *E. mixta* vs *E. bombiformis* | 0.0004 | | |
| *E. imperialis* vs *X. aeneipennis* | <0.0001 | | |
| *E. tridentata* vs *E. bombiformis* | <0.0001 | | |
| *E. tridentata* vs *E. frontalis* | 0.017 | | |
| *E. tridentata* vs *X. aeneipennis* | <0.0001 | | |
| *E. bombiformis* vs *P. orizabaensis* | 0.011 | | |
| *E. bombiformis* vs *T. ziegleri* | 0.0005 | | |
| *E. bombiformis* vs *T. angustula* | 0.0005 | | |
| *E. bombiformis* vs *T. fuscipennis* | 0.0003 | | |

| | |
|---|---|
| *E. frontalis* vs *T ziegleri* | 0.015 |
| *E. frontalis* vs *T. angustula* | 0.01 |
| *E. frontalis* vs *T. fuscipennis* | 0.008 |
| *E. frontalis* vs *X. aeneipennis* | <0.0001 |
| *P. orizabaensis* vs *X. aeneipennis* | <0.0001 |
| *T. ziegleri* vs *X. aeneipennis* | <0.0001 |
| *T. angustula* vs *X. aeneipennis* | <0.0001 |
| *T. fuscipennis* vs *X. aeneipennis* | <0.0001 |

**Table 2S.** Critical thermal minima ($CT_{Min}$) and maxima ($CT_{Max}$), intertegular distance (ITD), head width (HW), and average ovary size ($mm^2$) among females of *Megalopta* with different reproductive status. Mean value is followed by SE and sample size.

| **Reproductive status** | **$CT_{Min}$ (˚C)** | **$CT_{Max}$ (˚C)** | **ITD (mm)** | **HW (mm)** | **Ovary area ($mm^2$)** |
|---|---|---|---|---|---|
| Queen | 10.69 ±0.22, *N* = 13 | 41.47 ±0.28, *N* = 12 | 2.84 ±0.09, *N* = 13 | 4.06 ±0.14, *N* = 13 | 3.08 ±0.34, *N* = 13 |
| Worker | 9.53 ±0.26, *N* = 17 | 41.29 ±0.26, *N* = 17 | 2.61 ±0.08, *N* = 17 | 3.64 ±0.11, *N* = 17 | 1.55 ±0.34, *N* = 17 |
| Solitary reproductive | 9.92 ±0.17, *N* = 40 | 41.39 ±0.25, *N* = 31 | 2.81 ±0.04, *N* = 40 | 3.98 ±0.06, *N* = 40 | 2.67 ±0.18, *N* = 40 |

**Table 3S.** Cox proportional hazards estimates of the survival of nocturnal and diurnal bees after exposure to a heat stress event (38 ˚C) over 5 hours. *P*-values refer to comparisons with nocturnal bees. Significant values in boldface. HR = Hazard ratio; CI = Confidence interval. See Table 7S for species and sample size used in this experiment.

| **Bee** | **HR (95% CI)** | ***P*-value** |
|---|---|---|
| Nocturnal bees | — | — |
| Orchid bees | 1.15 (0.69–1.91) | 0.6 |
| Stingless bees | 0.41 (0.22–0.78) | **<0.01** |

**Table 4S.** *P*-values of pairwise comparisons among survival of nocturnal and diurnal bees using a Log-rank test after a heat stress event (38 ˚C) over 5 hours. Significant *P*-value in boldface. See Table 7S for species and sample size used in this experiment.

| | Nocturnal bees | Orchid bees | Stingless bees |
|---|---|---|---|
| Nocturnal bees | — | 0.49 | **<0.01** |
| Orchid bees | 0.49 | — | **<0.01** |
| Stingless bees | **<0.01** | **<0.01** | |

**Table 5S.** Survival time (hour) and percentage of water loss between nocturnal and diurnal bees exposed to a desiccant. Mean value is followed by SE and sample size. See Table 7S for species and sample size used in this experiment.

| Treatment | Diurnal bees | Nocturnal bees |
|---|---|---|
| | Survival time (Hour) | |
| Control | 14.77 ±2.20, $N = 30$ | 46.45 ±3.26, $N = 27$ |
| Desiccant | 14.87 ±1.47, $N = 30$ | 28.98 ±1.83, $N = 31$ |
| | Water loss (%) | |
| Control | 16.55 ±1.74, $N = 30$ | 21.88 ±2.63, $N = 23$ |
| Desiccant | 28.04 ±2.56, $N = 30$ | 22.35 ±1.77, $N = 24$ |

**Table 6S.** Results of pairwise comparisons with Bonferroni adjustment of the survival time and percentage of water loss of nocturnal and diurnal bees exposed to a desiccant. *DF* 1 in all comparisons. Significant *P*-value in boldface. See Table 7S for species and sample size used in this experiment.

| **Comparison** | **Survival time** | **Water loss** |
|---|---|---|
| Control diurnal vs Treatment diurnal | 1.000 | **0.001** |
| Control diurnal vs Control nocturnal | **<0.001** | 0.577 |
| Control diurnal vs Treatment nocturnal | **0.003** | 0.404 |
| Treatment diurnal vs Control nocturnal | **<0.0001** | 0.333 |
| Treatment diurnal vs Treatment nocturnal | **0.006** | 0.439 |
| Control nocturnal vs Treatment nocturnal | **<0.0001** | 1.000 |

**Table 7S.** Bee species used in the acute heat stress and desiccation stress assays (indicated with a plus sign). Number of individuals indicated in parentheses.

| **Species** | **Acute heat stress** | **Desiccation stress** |
|---|---|---|
| Diurnal bees | | |
| *Euglossa bursigera* Moure | + (1) | |
| *E. crassipunctata* Moure | + (2) | |
| *E. igniventris* Friese | + (1) | |
| *E. imperialis* Cockerell | + (11) | + (2) |
| *E. mixta* Friese | + (3) | |
| *E. tridentata* Moure | + (4) | |
| *Eulaema meriana* (Olivier) | + (15) | |
| *Partamona orizabaensis* (Strand) | + (6) | + (14) |
| *Plebeia franki* (Friese) | + (4) | |
| *Tetragona ziegleri* (Friese) | + (17) | + (9) |
| *Tetragonisca angustula* (Latreille) | + (1) | + (15) |
| *Trigona fulviventris* | | + (7) |
| *Trigona fuscipennis* Friese | | + (13) |
| **Nocturnal bees** | | |
| *Megalopta amoena* (Spinola) | + (2) | + (10) |
| *M. genalis* Meade-Waldo | + (33) | + (48) |

**Figure 1S.** Temperature (a) and relative air humidity at the forest understory where nests of *Megalopta* are commonly found (~1 m above ground). Box plots show median, quartiles, and extreme values. For each figure, a different letter above bars indicates significant differences ($P < 0.05$).

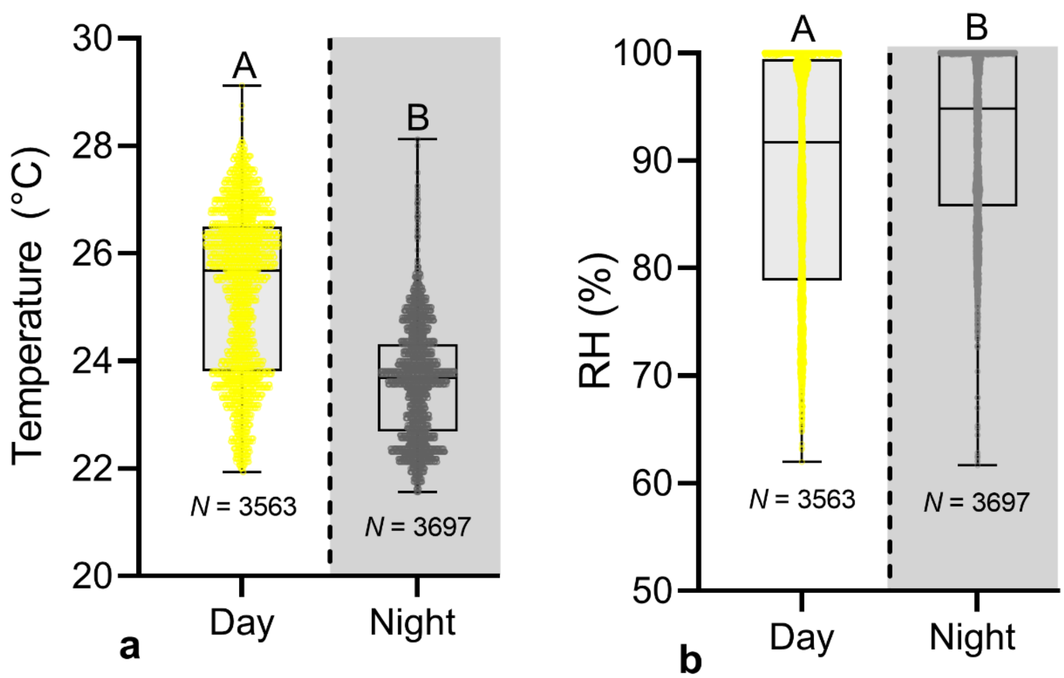


**Figure 2S.** Ambient and internal nest temperature of nocturnal sweat bees of the genus *Megalopta*. a) Box plots showing median, quartiles, and extreme values of temperatures. Groups with different letters are significantly different ($P < 0.05$). b) Changes in temperature at 5 min intervals during a 4 h period, from 9:30 to 13:30 h.

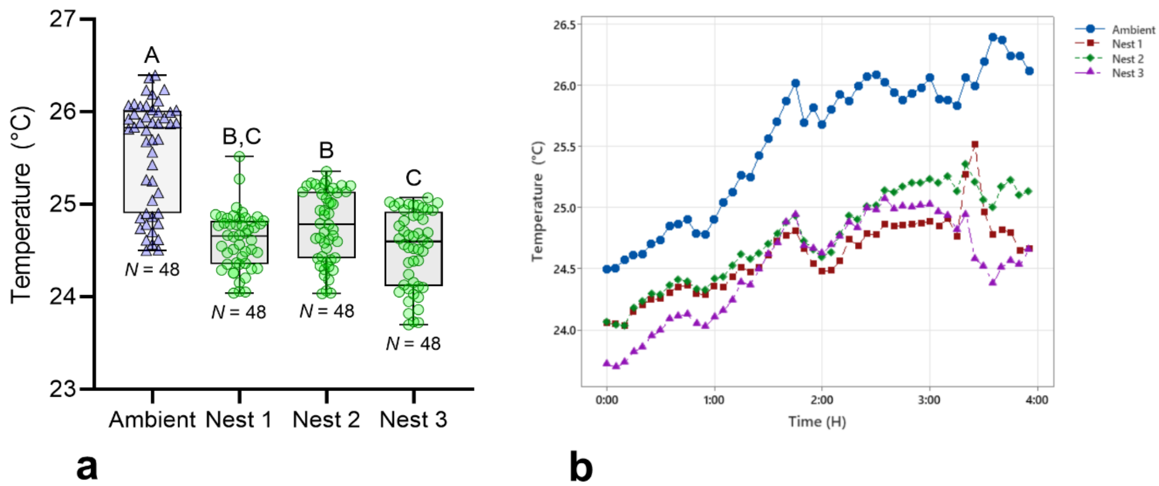


**Figure 3S.** Box plots showing $CT_{Min}$ and $CT_{Max}$ among species of diurnal and nocturnal bees. Species are organized into broader taxonomic groups (carpenter bees, orchid bees, stingless bees, and nocturnal bees). For each plot, a different capital letter above taxonomic group indicates significant differences ($P < 0.05$). To facilitate comparisons, a horizontal line was placed at 10 °C in the $CT_{Min}$ plot and at 45 °C in the $CT_{Max}$ plot.

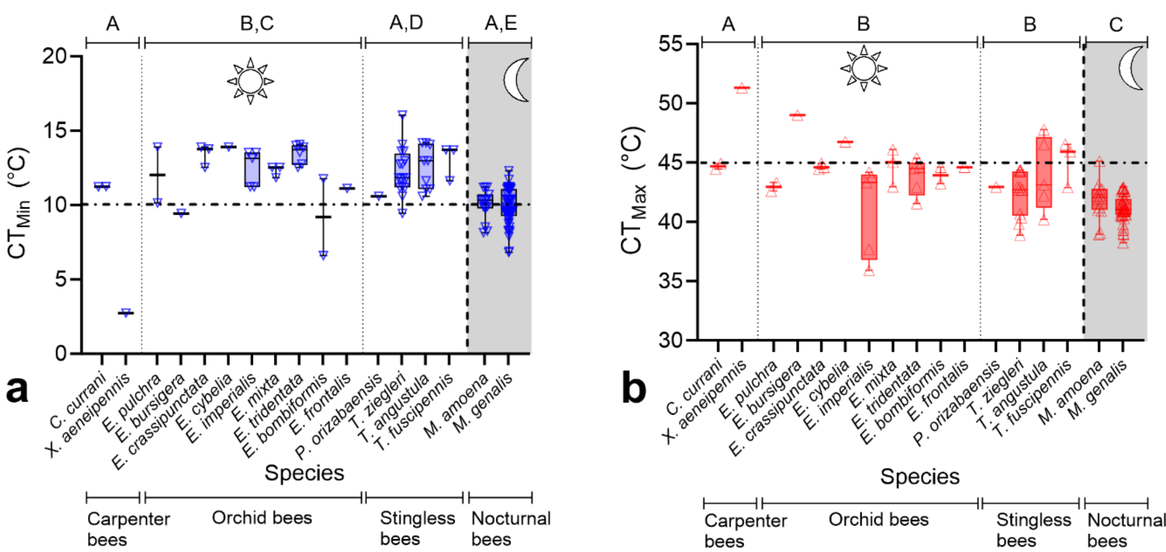


**Figure 4S.** Critical thermal minima ($CT_{Min}$) and maxima ($CT_{Max}$) and their relationship with maximum head width (HW) and average ovary area in nocturnal bees.

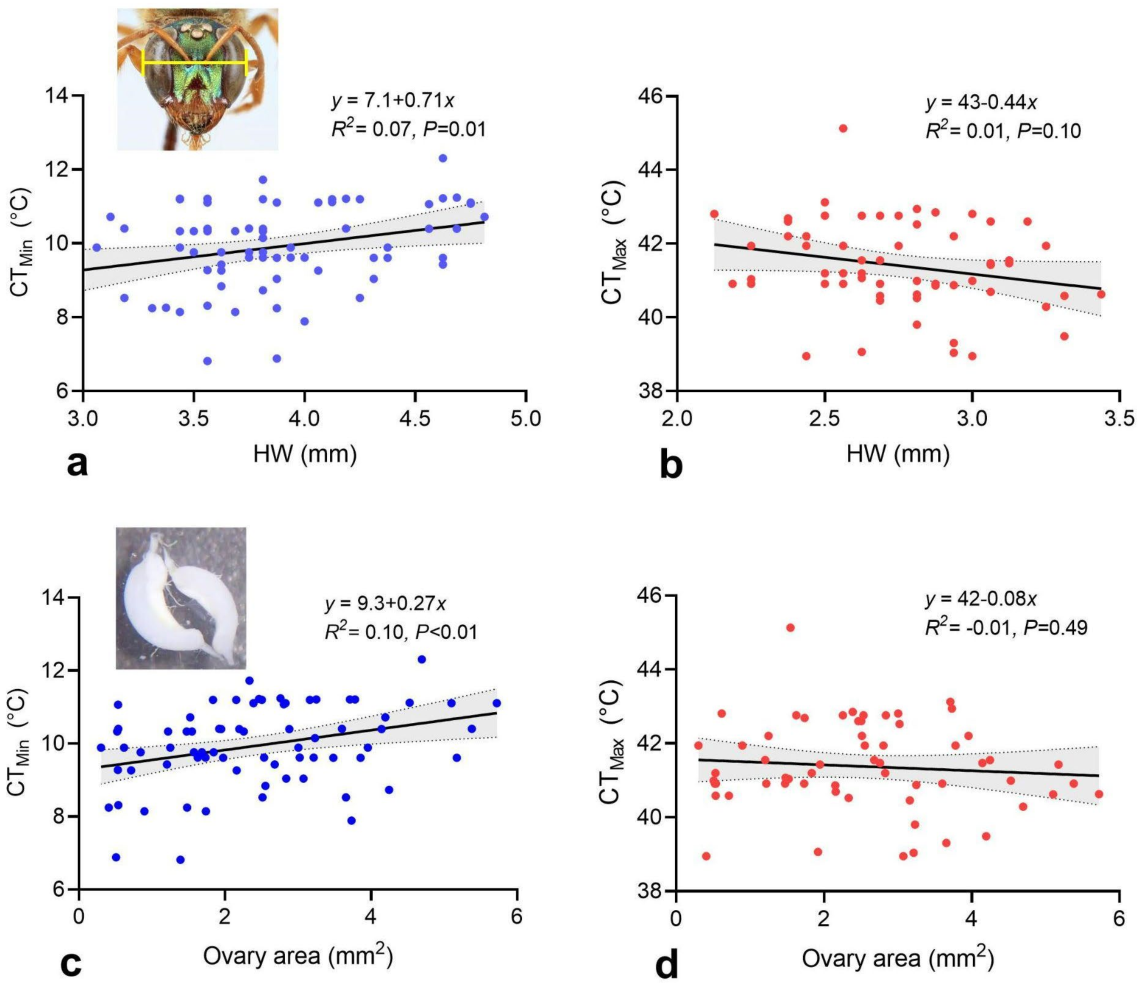


**Figure 5S.** Critical thermal minima ($CT_{Min}$) and maxima ($CT_{Max}$) among females of *Megalopta* with different reproductive status. Box plots show median, quartiles, and extreme values of temperatures. For each trait, groups with different letters are significantly different ($P < 0.05$).

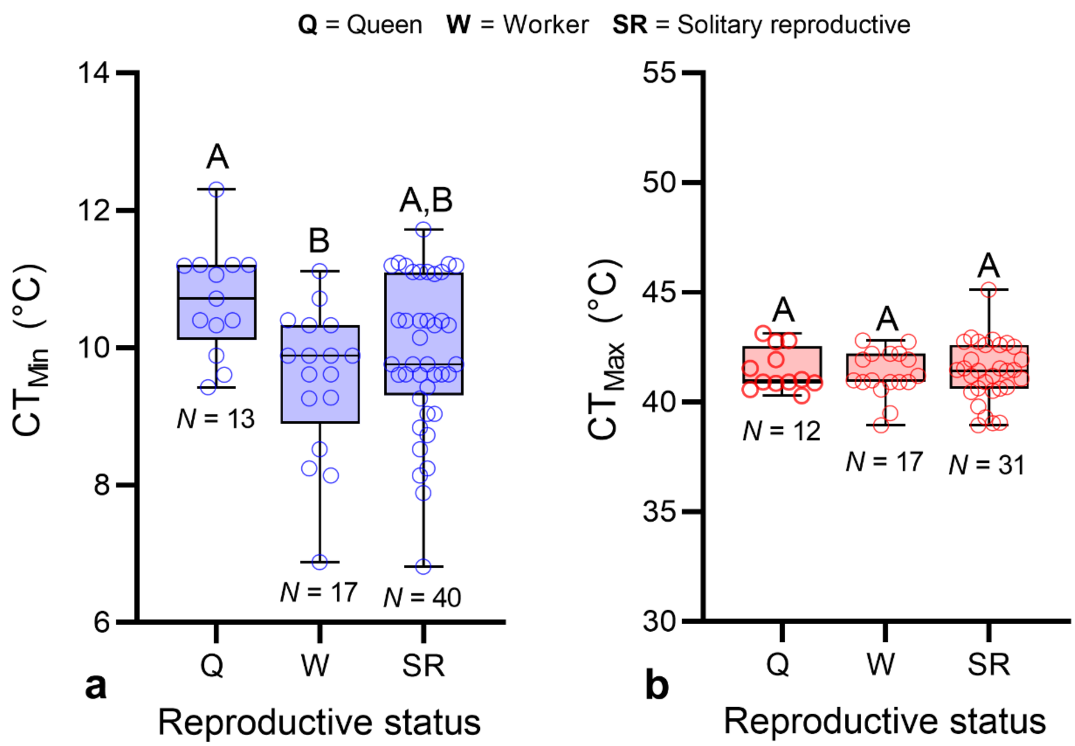


**Figure 6S.** Relationship between survival time of bees exposed to a desiccant and intertegular distance (ITD).

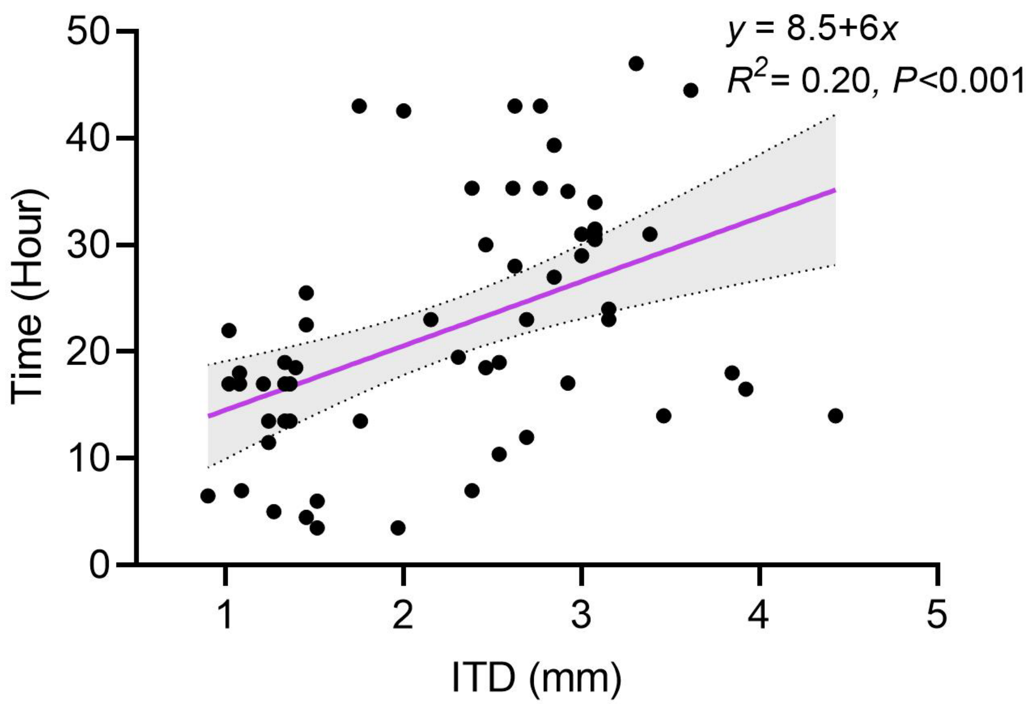


**Fig 7S.** Phylogenetic reconstruction of focal species. Node support indicated by the SH-aLRT value followed by the ultrafast bootstrap value after 1,000 replicates.

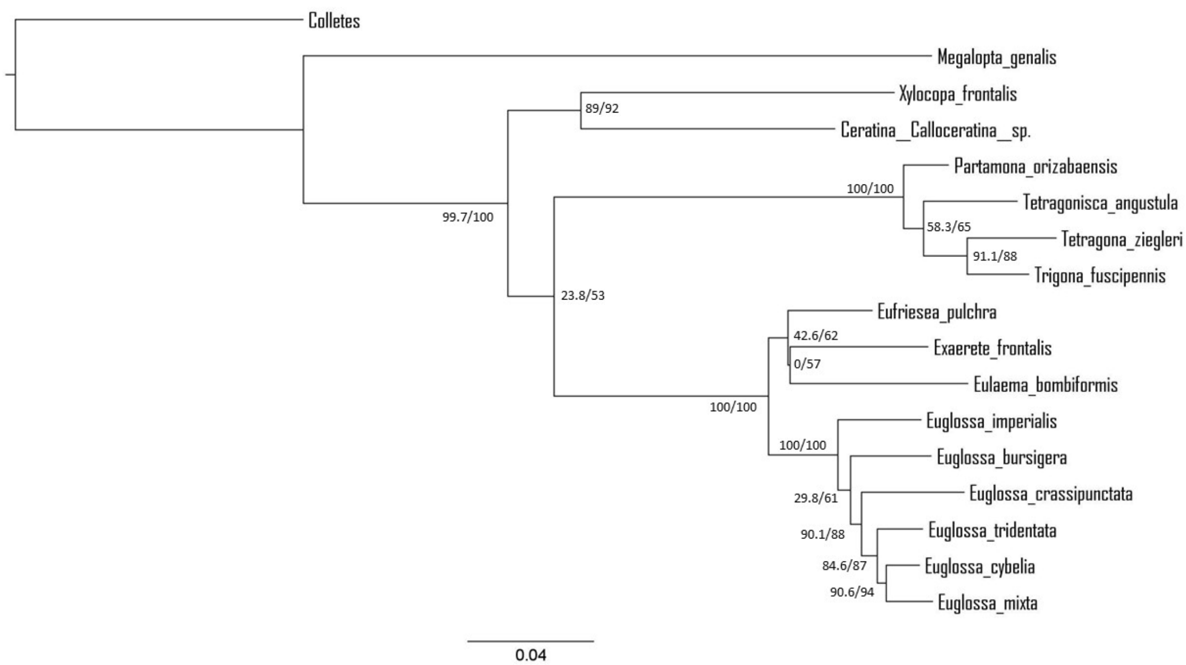



**Fig 8S.** Phylogenetic reconstruction of the focal bee species used in this study and their critical thermal minima ($CT_{Min}$).

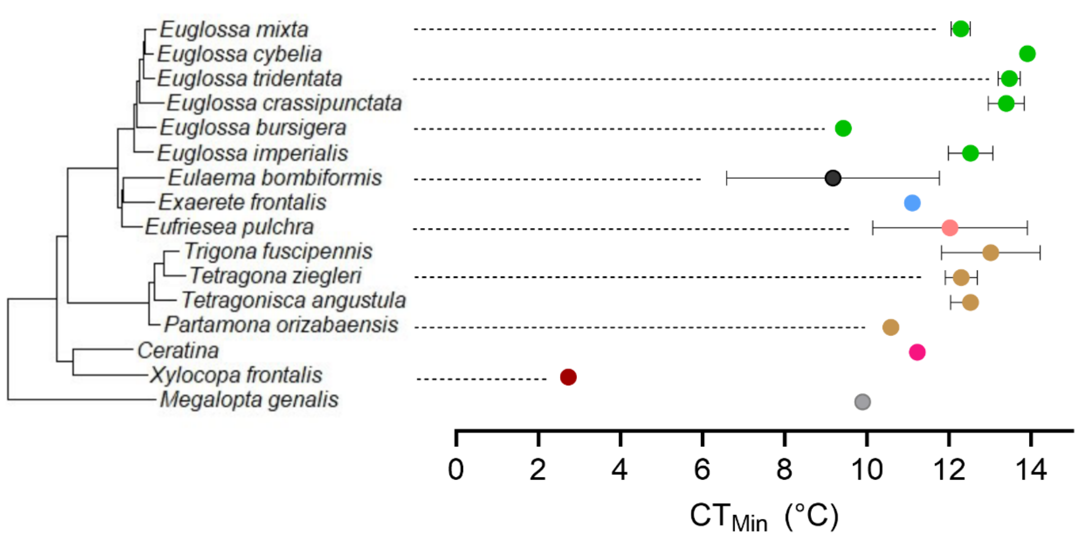
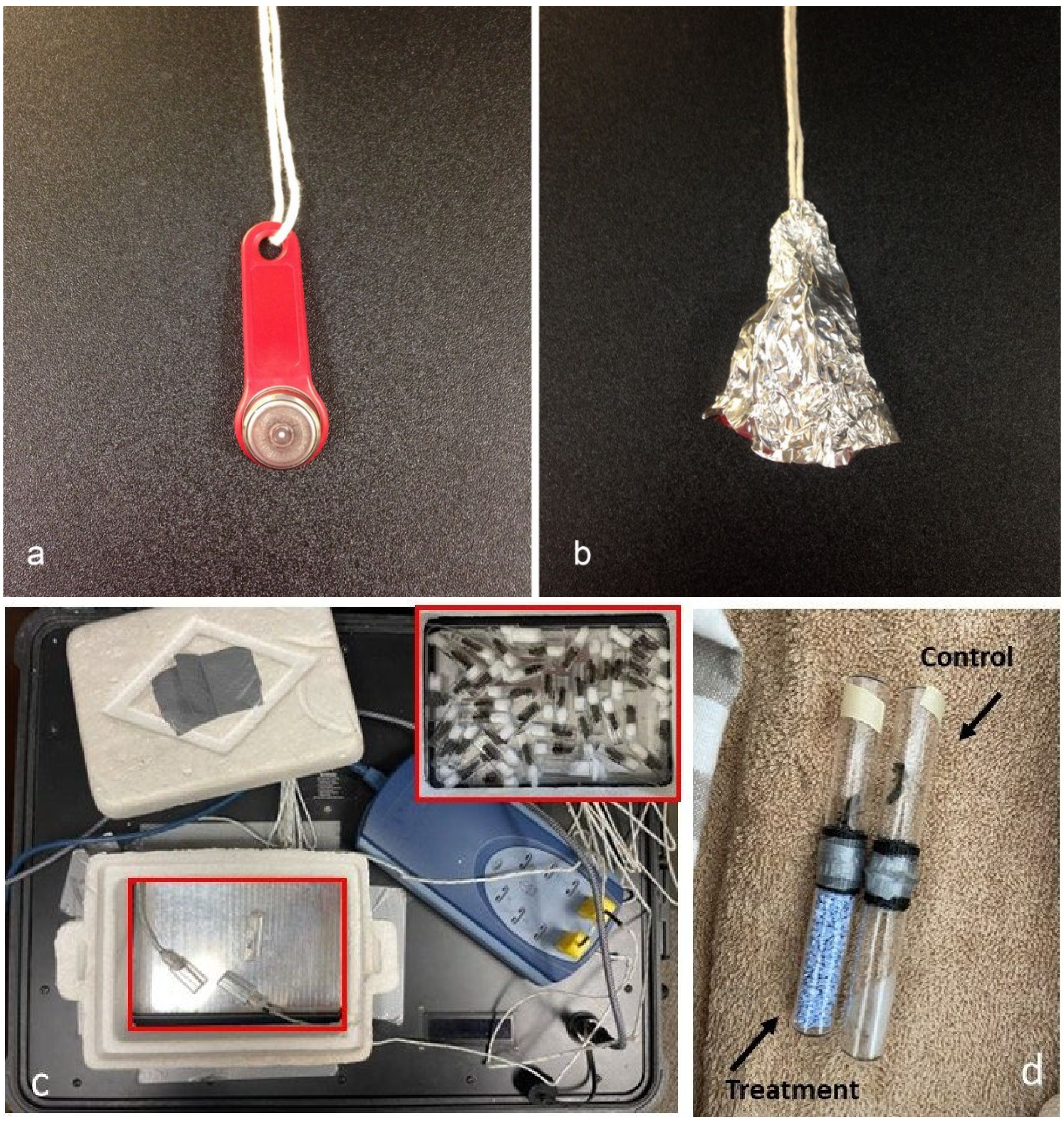


**Figure 9S.** Temperature and humidity sensor used in the field, thermal equipment, and desiccation apparatus. **a** = Thermochron Fob (red plastic holder) and iButton sensor. **b** = Plastic holder and iButton shielded with a piece of aluminum foil (~12 × 7 cm). **c** = Elara 2.0, a portable fully programmable heating/cooling anodized aluminum stage (outlined in red) designed for precision temperature control. Insert in upper right corner shows testing vials with bees, which are plugged with a moisten cotton ball. **d** = desiccation apparatus filled with fully dehydrated Drierite desiccant (treatment, left tubes) or with a moistened paper towel (control, right tubes).



**Supplementary methods**

***Phylogenetic inference***. We used sequences available through GenBank[1] for the following five nuclear and mitochondrial gene fragments: arginine kinase (AK), elongation factor 1-α (EF1α), cytochrome oxidase I (COI), cytochrome b (CytB), and the 28S rDNA. We chose *Colletes inaequalis* Say as the outgroup. Ingroup sampling included sequences available for our focal species or their closest relative, as in the case of *X. aeneipennis*. We used Geneious prime® 2023.0.4 (http://www.geneious.com/) to align and concatenate sequences. We aligned sequences using the MAFFT Alignment option with default settings[2], with a final concatenated alignment length of 4,504 bp. We inferred trees with the Maximum likelihood (ML) software IQ-TREE[3] 1.6.12 through the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/). For ML inference we partitioned the concatenated dataset *a priori* by marker (*i.e.*, each marker composing a single data subset). Substitution models were searched using the "Auto" option and the edge-proportional partition model. We assessed branch support with 1,000 Ultrafast bootstrap replicates[4] and single branch tests via SH-like approximate likelihood ration test (SH-aLRT[5]). Trees were visually inspected and annotated in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/).

**References**

1. Benson, D.A. *et al*. GenBank. *Nucleic Acids Res.* **41**, D36-D42 (2012).
2. Katoh, K. & Standley, D.M. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. *Mol. Biol. Evol*. **30**, 772–780 (2013).
3. Nguyen L-T, Schmidt, H.A., von Haeseler, A. & Minh, B.Q. IQ-TREE: A fast and effective stochastic algorithm for estimating Maximum-likelihood phylogenies. *Mol. Biol. Evol*. **30**, 268–274 (2015).
4. Minh, B.Q., Nguyen, M.A.T. & Von Haeseler, A. Ultrafast approximation for phylogenetic bootstrap. *Mol. Biol. Evol*. **30**, 1188–1195 (2013).
5. Guindon, S. *et al*. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321 (2010).

**Table 8S.** GenBank accession numbers for sequences used in this study.

| Species in this study | Species in Genebank | AK | EF1α | COI | CytB | 28S |
|---|---|---|---|---|---|---|
| | *C. inaequalis* | — | AY585123 | EF028488 | — | AY654484 |
| *C. currani* | *C.* (*Calloceratina*) sp. | — | GU321639 | JX968036 | GU321574 | — |
| *E. pulchra* | *E. pulchra* | EU421637 | EU421377 | EU421506 | — | HM750234 |
| *E. bursigera* | *E. bursigera* | EU421701 | EU421446 | EU421573 | AY916113 | — |
| *E. crassipunctata* | *E. crassipunctata* | EU421626 | EU421365 | EU421494 | AY916120 | — |
| *E. cybelia* | *E. cybelia* | EU421645 | EU421386 | AY506430 | AY916110 | — |
| *E. imperialis* | *E. imperialis* | EU421668 | EU421408 | EU421537 | AY916117 | HM750232 |
| *E. mixta* | *E. mixta* | — | EU421436 | EU163094 | AY916106 | — |
| *E. tridentata* | *E. tridentata* | EU421702 | EU421447 | EU421574 | AY916105 | — |
| *E. bombiformis* | *E. bombiformis* | EU421655 | EU421395 | EU421524 | AF002728 | — |
| *E. frontalis* | *E. frontalis* | AY267175 | AY267143 | EU421478 | AY916098 | HM750233 |
| *P. orizabaensis* | *P. orizabaensis* | FJ042241 | FJ042342 | — | — | FJ042066 |
| *T. ziegleri* | *T. ziegleri* | — | FJ042387 | AF066991 | AF002725 | FJ042115 |
| *T. angustula* | *T. angustula* | — | FJ042381 | MN344929 | KF891236 | FJ042109 |
| *T. fuscipennis* | *T. fuscipennis* | EU184829 | EU184770 | — | — | HM750238 |
| *X. aeneipennis* | *X. frontalis* | — | JQ230049 | KC853310 | AY005275 | — |
| *M. genalis* | *M. genalis* | — | AF140316 | JQ266453 | — | JQ279190 |