Heatwave length and severity drive development disruption in a key pollinator

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Abstract

Heatwaves are becoming more frequent and severe due to climate change, posing a significant threat to global biodiversity and entire ecosystems. Despite alarming projections of population declines for key pollinators like bumblebees, crucial for crop pollination and ecosystem services, surprisingly little is known about the implications of heatwaves on their larval and pupal development. Here, we experimentally exposed *Bombus terrestris* L. (Hymenoptera: Apidae) pupae *in vitro* to varying heatwave scenarios in terms of length (3, 5, or 7 days) and intensity (36°C, 37°C, and 38°C) to study the impact on their development at this critical life stage. We assessed their emergence rate, developmental duration, lipid content, sex-specific body size and wing geometrics morphometrics. Our major findings revealed a critical heat stress threshold at 38°C, where pupal emergence was drastically reduced by almost twofold and fivefold for 3 and 5 days, respectively. Adults emerged from these heatwave treatments exhibited a fourfold and sevenfold increase in wing deformities. Even excluding these extreme cases, we found clear evidence for heatwave-induced effects on female wing shape and directional asymmetry (DA) among treatments. Given the relative heat tolerance of our model species *B. terrestris*, we expect even greater vulnerability in more cold-adapted bumblebee species. Our findings underscore the profound ecological consequences of heatwaves on bumblebee pupal development, with potentially devastating cascading effects on colony fitness, population viability, crop pollination, and ultimately, ecosystem services essential for human well-being.

Keywords social insect, wild bees, extreme heat, acute stress, metamorphosis, malformations

1. Introduction

Global warming is undoubtedly one of the most pressing challenges of our time (Garcia, Cabeza, Rahbek, & Araújo, 2014; IPCC, 2023). Particularly the increase in frequencies and intensities of extreme weather events such as heatwaves are concerning (Lhotka, Kyselý, & Farda, 2018; Meehl & Tebaldi, 2004; Perkins-Kirkpatrick & Lewis, 2020; Stillman, 2019). Heatwaves, defined as periods of abnormally hot temperatures of at least three consecutive days, can severely affect the life of wide range of terrestrial animals such as insects, birds and mammals (González-Tokman et al., 2020; Ma, Ma, & Pincebourde, 2021; Stillman, 2019). Despite recent advances in understanding the impact of heatwaves, our knowledge of their impact on animal development, their fitness and entire populations is still limited (González-Tokman et al., 2020; Jentsch, Kreyling, & Beierkuhnlein, 2007; Ma et al., 2021; Stillman, 2019).

Gaining a better understanding of how the length and severity heatwaves impact animal physiology and fitness might be particularly important in threatened key-stone species such as bumblebees (*Bombus* sp., Hymenoptera: Apidae). Bumblebees provide crucial pollination services in ecosystems and agriculture in the Northern hemisphere (Goulson, Lye, & Darvill, 2008; Klein et al., 2007). As cold-adapted taxon, bumblebees appear to be particularly vulnerable to climate change, which is reflected in their alarming population declines in some species (Cameron et al., 2011; Cameron & Sadd, 2020; Ghisbain et al., 2024; Kerr et al., 2015; Maebe et al., 2021; Martinet et al., 2021; Potts et al., 2010; Soroye, Newbold, & Kerr, 2020). However, heat tolerance varies significantly across species, with alpine and polar species being especially vulnerable to heat stress (Martinet et al., 2021a; Zambra et al., 2020). In contrast, commercially used bumblebees like *Bombus terrestris* L. are commonly kept aboveground in greenhouses as crucial crop pollinators (Potts et al., 2016), which puts them at increased risk of exposure to extreme heat. Previous studies have shown that exposure to elevated heatwave-like temperatures impairs cognition (Gérard, Amiri, Cariou, & Baird, 2022) and scent perception (Nooten, Korten, Schmitt, & Kárpáti, 2024) in worker bumblebees. Under extreme heat stress, the

survival is significantly affected (Kuo, Lu, Lin, Lin, & Wu, 2023; Quinlan, Feuerborn, Hines, & Grozinger, 2023). Additionally, increasing temperatures negatively correlate with colony growth, which in turn negatively affects gyne production (Theodorou et al., 2022).

Colony fitness may not only be influenced by quantity of offsprings (Theodorou et al., 2022), but also by their quality, expressed in their phenotypic traits. In particular, wing morphology (shape, size and asymmetry) and storage reserves (e.g. fat body size) have been suggested as potential markers for monitoring bee health and pollination service, as they correlate with reproductive success, immunity, resilience and foraging efficiency (Vanderplanck, Breeze, Cini, Potts, & Senapathi, 2021). Indeed, experimental exposure of bumblebee colonies to prolonged constant heat led morphological changes in their offspring, including alterations in wing morphology, reduced body size and smaller antennae (Guiraud, Cariou, Henrion, Baird, & Gérard, 2021; Gérard, Cariou, Henrion, Descamps, & Baird, 2022; Gérard, Guiraud, Cariou, Henrion, & Baird, 2023; Gérard et al., 2018; Perl et al., 2022). Moreover, elevated nest temperatures during their larval development seem to have carry-over effects on adult behaviour (Gérard, Cariou, et al., 2022; Perl et al., 2022). To prevent or limit the negative consequences of elevated nest temperatures workers spend significantly more time fanning to cool the brood (Bretzlaff, Kerr, & Darveau, 2024; Sepúlveda, Nicholls, Schuett, & Goulson, 2024). While different developmental stages are likely differentially affected by heat stress, it remains unclear which stages are most vulnerable and if short-term heatwaves are sufficient to impair their development.

To study the impact of heatwave length and sevirity on pupal development in bumblebees, we used an *in vitro* rearing protocol (Kato, Kikuta, Barribeau, & Inoue, 2022; Woegler & Kurze, 2024), isolating confounding influences of the worker behaviour in the colony. We hypothesised that an increase in rearing temperatures and prolonged exposure would adversely affect the emergence rate, developmental duration, adult body size, fat body size, and wing development.

2. fMethods

(a) Experimental overview

To study the impact of heatwave length and sevirity on pupal development in bumblebees, we collected 887 fourth instar larvae from 25 commercial *Bombus terrestris* colonies to rear them *in vitro* following previous protocols (Kato et al., 2022; Woegler & Kurze, 2024). While our *in vitro* rearing temperature of 34°C (Kato et al., 2022) exceeds typical nest temperatures, it aligns with brood temperatures, which are typically 2°C warmer than ambient nest temperatures (Vogt, 1986; Weidenmüller, Kleineidam, & Tautz, 2002). With the beginning of the pupal stage, they were pseudo-randomly assigned to one of the seven treatment groups to ensure relative equal distribution among treatments and colonies. Pupae were exposed to either 36°C for 5 day (36C5d, n = 79), 36°C for 7 days (36C7d, n = 80), 37°C for 3 day (37C3d, n = 75), 37°C for 5 days (37C5d, n = 73), 38°C for 3 day (38C3d, n = 64), and 38°C for 5 days (38C5d, n = 106), while the controls (Ctrl/34C, n = 127) were kept at a constant 34°C. We checked their survival until adult bees emerged and measured their body mass. If adult bees emerged, we freeze killed them at age of 2 days and kept them at -20°C until subsequent morphometric measurements of their head width, intertegular distance (ITD), dry mass, and wings.

(b) Colony husbandry

Larvae were collected from 25 queen-right colonies of *Bombus terrestris* (Natupol Research Hives, Koppert B.V., Netherlands) at various colony phases. Colonies were maintained under conditions similar to our previous studies (Gilgenreiner & Kurze, 2024; Woegler & Kurze, 2024). In short, they were housed in the standard Natupol nest boxes (23 (l) x 25 (w) x 15 (h) cm), which were connected to foraging arenas (59 (l) x 39 (w) x 26 (h) cm) under a 14:10 h light:dark cycle. Bumblebees had *ad libitum* access to 70% (w/v) sucrose solution. Depending on colony size, each colony received 3-11 g of pollen candy daily, which consisted of 67% organic pollen (naturwaren-niederrhein GmbH, Germany),

25% sucrose (Südzucker AG, Germany), and 8% tap water. The room temperature was maintained at 25 °C ± 1 °C and 30-50% relative humidity.

(c) Larvae collection and *in vitro* rearing

To collect 4th instar larvae (L4), all adult bees from a colony were temporarily transferred to a separate cage. Then, L4 larvae were carefully removed from individual, spherical brood cells using soft tweezers. L4 larvae can be easily identified by the small opening for food provisioning (figure S1a) (Woegler & Kurze, 2024). To minimize disturbance, colonies were only resampled after minimum period of at least two weeks.

Depending on the size of L4 larvae, they were carefully transferred into smaller or larger 3D-printed polylactide (PLA) artificial brood cells (capacity of either 0.6 mL or 0.96 mL, diameter of either 8 mm or 10 mm respectively, figure S1b). Those were then placed in 24- or 48-well clear flat bottom plates (Falcon/Corning, USA) (figure S1c) and kept inside a ventilated plastic container (18.5 x 18.5 x 11.5 cm) at 34°C (KB115, BINDER GmbH, Germany). A 120 mL cup of saturated sodium chloride solution was included within each container to maintain a relative humidity of 65 ± 10 %. The use of this setup facilitated the measurement of larval and pupal weight gain without the need for repeated handling them directly and allowed for the easy removal of any dead individuals.

L4 larvae were fed a pollen medium twice daily (morning and evening) until pupation and the start of the experiment. The pollen medium consisted of 50% w/v sucrose solution (Südzucker AG, Germany), 40% honeybee-collected organic pollen (Bio-Blütenpollen, naturwaren-niederrhein GmbH, Germany), 10% Bacto yeast extract (Bacto™, BD, USA), and 1% casein sodium salt from bovine milk (Sigma-Aldrich, Germany) (Kato et al., 2022). The pollen medium was aliquoted and stored at -20°C until use. Before hand-feeding all L4 larvae, aliquots were warmed to 34°C and vortexed. For each 20-minute feeding session, well-plates containing the L4 larvae were placed on a heated plate at 35°C (Medax model 12801, Medax Nagel GmbH, Germany). Each larva initially received about 6 µL droplet (7.1 ± 1.6 mg, 10 µL micropipette tips had to be cut off at the 1 µL mark) of pollen medium applied to its ventral abdomen (figure S1d). Larvae

that consumed all initial food received a second droplet. Satiation was determined when a larva curled up and stopped movement, while active larvae were considered hungry (Supplementary video 1). At the end of each feeding session, any remaining food was carefully removed to prevent tracheal blockage. Larvae were no longer fed when entering the pupal stage.

At the start of pupal stage, individuals were weighed (d = 0.1 mg, Sartorius AC120S, Sartorius AG, Germany) and pseudo-randomly assigned to one of seven treatment groups, ensuring a balanced distribution across treatments and natal colonies. Pupae were exposed to one of the following heatwave scenarios: 36°C for 5 day (36C5d, n = 79), 36°C for 7 days (36C7d, n = 80), 37°C for 3 day (37C3d, n = 75), 37°C for 5 days (37C5d, n = 73), 38°C for 3 day (38C3d, n = 64), or 38°C for 5 days (38C5d, n = 106). Controls (Ctrl/34C, n = 127) were reared at a constant 34°C. Pupae were transferred to new humidity-controlled containers inside an incubator set to the designated temperature. After the treatment period, they were returned to the control incubator at 34°C. Although pupal survival was recorded daily, these data are not presented due to the difficulty in accurately determining viability in the pupae. Instead, we decided to report the proportion of larvae that successfully reached adulthood.

(d) Emerged adult bees

For each bee reaching adulthood, the emergence date was recorded to calculate the developmental requited to complete metamorphosis (i.e. pupal phase). Their body weight was directly measured using a fine scale (d = 0.1 mg, Sartorius AC120S, Sartorius AG, Germany). Adult bees were then housed individually in 25 mL snap-cap vial containing a cardboard bottom (approximately 5.5 x 1.5 cm) and mesh lid. Bees had *ad libitum* access to 60% (w/v) sugar solution via simple 1.5 mL feeder and kept within the same rearing room where their natal colonies were maintained. Two-day-old adults, with fully developed and hardened wings, were then euthanized by freezing and stored at -20°C until further analysis.

(e) Body size and lipid measurements

As a proxy for adult body size, the intertegular distance (ITD) and head width were measured using a digital microscope (VHX-500F, Keyence GmbH, Germany) (Gilgenreiner & Kurze, 2024). In addition, their sex was determined by counting antennal segments (females have 12, males 13). To determine adult dry mass, each corpse was carefully dissected by one cut at ventral abdominal segments, then dried at 60°C for three days (U40, Memmert GmbH & Co. KG, Germany), and weighed (d = 0.1 mg, analytic balance M-Pact AX224, Sartorius GmbH, Germany). Subsequently, the body lipids were extracted using petroleum ether for five days. The ether was discarded, and the corpses were rinsed with fresh ether before being dried for an additional three days. The lipid content was calculated as the difference between the initial and post-extraction dry masses. The relative lipid content, serving as a proxy for the fat body size, was calculated as the percentage of lipid contents relative to the initial dry masses.

(e) Wings

Wings were first detached at the tegulae and embedded side-by-side on a microscope slide (Roti®Mount, Carl Roth GmbH + Co. KG, Germany). Then, wing pairs were photographed at 20x magnification (Keyence VHX-500F). Two-dimensional coordinates of twenty landmarks for each wing (figure S1e) were obtained using TpsDig2 (v2.32) (Rohlf, 2015). While individuals with visibly undeveloped or fully deformed wings (figure S1f) were excluded from geometric morphometric analysis, we compared the proportion of individuals with developed, undeformed wings among treatment groups.

(g) Statistical analyses

All statistical analyses and data visualizations were performed using R Statistical Software in R version 4.4.2 (R Core Team, 2024). The complete code and output are provided in the electronic supplementary material.

Generalized linear mixed effect models (GLMMs) using the *glmmTMB* package (Brooks et al., 2017) were used to analyse effects of the treatment length and intensity

(temperature), and their interactions, as fixed factors on emergence rates (i.e. pupae reaching adulthood), developmental time (i.e. days to emergence), adult mass, dry mass, ITD, head width, and wing development (i.e. either fully developed or deformed) with binomial (logit link) or gamma (log) data structures. We included ‘colony ID’ and ‘initial pupae mass’ as random effects to account for colony and larval-specific variability. Additionally, we analysed the effects on adult mass, dry mass, ITD, and head width for female (i.e. worker) and male data separately, to account for any sex-specific biases.

Model selection was performed based on the Akaike information criterion (AIC) and likelihood ratio tests. The final models were compared with their respective null-models. Model assumptions and dispersion of the data were checked using the *DHARMa* package (Hartig, 2020). Significance levels ($p < 0.05$) were determined using the *Anova* function when assumptions were met. Contrast pairwise comparisons between each treated group with the controls were conducted using the function *emmeans* (Lenth & Lenth, 2018) adjusted with Holm-Bonferroni correction. When model assumptions were not met, treatment effects were tested with a Kruskal-Wallis rank sum test, followed by post-hoc comparisons between each treated group with the controls using Dunn’s test with Holm-Bonferroni correction.

Geometric morphometric analyses of wing size, shape, and asymmetry on fully developed left and right forewings from females and male were performed separately on the obtained TPS data using geomorph package (Adams, Collyer, Kaliontzopoulou, & Baken, 2024), following a similar approach as previous studies (Aytekin, Terzo, Rasmont, & Çağatay, 2007; Gérard et al., 2018). Briefly, right wings were first mirrored to ensure landmark correspondence. Missing landmark data, representing morphological variation, were accounted for using the function *estimate.missing*. To remove non-shape variation, a Generalized Procrustes Analysis (GPA) was performed using *gpagen*. A Principal Component Analysis (PCA) was conducted on the Procrustes coordinates using *gm.prcomp* and PCA scores (PC1 and PC2) calculated and data distribution was visually assessed. Wing with PC1 z-scores exceeding $\pm$ 2 standard deviations were removed as outliers, where PC1 explained 71% of the data’s variation. Treatment groups with fewer

than six individuals were also excluded from further analysis. Then, centroid size and mean centroid size were calculated for each wing and each bee, respectively, as a measure of wing size. To analyse shape variation, a permutational multivariate analysis of variance (PERMANOVA) was performed using the *adonis2* function. Homoscedasticity was evaluated using *betadisper*, and pairwise comparisons were conducted using *pairwise.adonis* with Holm-Bonferroni correction. Directional asymmetry (DA) was calculated as the difference between left and right wing Procrustes coordinates, and DA magnitude was calculated as the mean Euclidean distance between corresponding landmarks. To assess fluctuating asymmetry (FA), a bilateral symmetry analysis was performed using *bilat.symmetry* and calculating the FA magnitude as the Euclidean norm of the FA component. The effects of treatment on centroid size, DA and FA magnitude were tested using linear regression models (LM) when assumptions were met, followed by contrast pairwise comparisons for significant results as described above. Kruskal-Wallis tests were used as non-parametric alternative, followed by Dunn's post-hoc test to compare treatment groups to the control by applying Holm-Bonferroni corrections.

3. Results

We found a strong interaction effect of heatwave temperature and length on the proportion of pupae reaching adulthood (GLMM: $\chi^2 = 23.056$, df = 1, $p < 0.0001$; figure 1a). Pupae of treatment 38C3d and 38C5d were 43% ($z = 3.523$, $p < 0.01$) and 83 % ($z = 6.563$, $p < 0.0001$) less probable to reach adulthood compared to controls, respectively. Heatwave intensity also significantly affected pupal developmental duration (Kruskal-Wallis: $\chi^2 = 69.584$, df = 6, $p < 0.0001$; figure 1b; female and male data presented separately in figure S2). While an exposure to 36°C shortened development compared to controls (Dunn's test: 36C5d, $z = 3.253$, $p < 0.01$; 36C7d, $z = 3.142$, $p < 0.01$), an exposure to 38°C prolonged development (38C3d, $z = -4.393$, $p < 0.0001$; 38C3d, $z = -2.739$, $p < 0.01$). There was no significant effect found for the 37°C exposure compared to controls (37C3d, $z = -1.361$, $p > 0.5$; 37C5d, $z = -1.164$, $p > 0.5$).

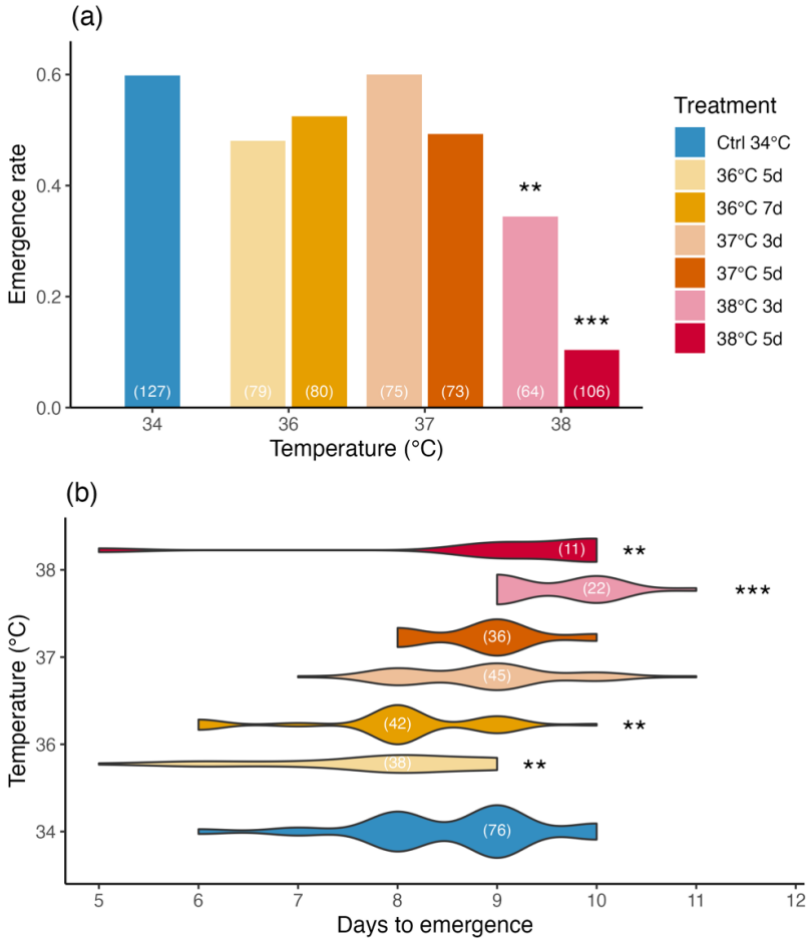

Figure 1. Direct effects of heatwaves of varying intensity on (a) emergence rate and (b) developmental duration in *B. terrestris* pupae. Controls (Ctrl, reared at constant 34°C, blue) were compared to individuals exposed to increased temperatures: of 36°C for 5 days (light warm yellow) and 7 days (warm yellow), 37°C for 3 days (light reddish-orange) and 5 days (reddish orange), and 38°C for 3 days (light red) and 5 days (deep red). Sample sizes are indicated by white numbers and significant differences are marked by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

When testing the effects of heatwave treatments on adult mass, we found a significant effect (Kruskal-Wallis: $\chi^2 = 30.552$, df = 6, $p < 0.0001$) with 36C7d pupae resulting in significantly lighter adults compared to controls (Dunn's test: $z = 2.576$, $p < 0.05$) and 38C3d pupae resulting in heavier adults ($z = -2.679$, $p < 0.05$). However, sex-specific analyses revealed no significant treatment effects on adult mass (Kruskal-Wallis: females, $\chi^2 = 9.125$, df = 6, $p > 0.05$, figure 2a; males, $\chi^2 = 6.526$, df = 6, $p > 0.05$, figure S3a). While there was a significant effect on female head width (Kruskal-Wallis: χ^2 =

16.131, df = 6, $p < 0.05$, figure 2b), we found no significant differences between treatment groups and controls (Dunn's tests: $p > 0.05$). However, female head width varied with temperature (Kruskal-Wallis: $\chi^2 = 12.311$, df = 3, $p < 0.01$), where adults from pupae exposed to 38°C had slightly wider heads than controls ($z = -2.227$, $p < 0.05$). We found no significant effect on female ITD (Kruskal-Wallis: $\chi^2 = 9.571$, df = 6, $p > 0.05$, figure 2c). Similarly, we also found no significant effects on male body size proxies (Kruskal-Wallis: head width, $\chi^2 = 9.532$, df = 6, $p > 0.05$, figure S3b; ITD, $\chi^2 = 4.743$, df = 6, $p > 0.05$, figure S3c).

In contrast, we found that heatwave intensity significantly influenced relative lipid content in adult females (Kruskal-Wallis: $\chi^2 = 25.069$, df = 6, $p < 0.0001$, figure 2d), but not in males (Kruskal-Wallis: $\chi^2 = 8.287$, df = 6, $p > 0.05$, figure S3d). We found an increase in relative lipid content in adult females of 37C5d (Dunn's test: $z = -2.592$, $p < 0.05$), 38C3d ($z = -2.844$, $p < 0.05$), and 38C5d ($z = -3.012$, $p < 0.01$) compared to controls.

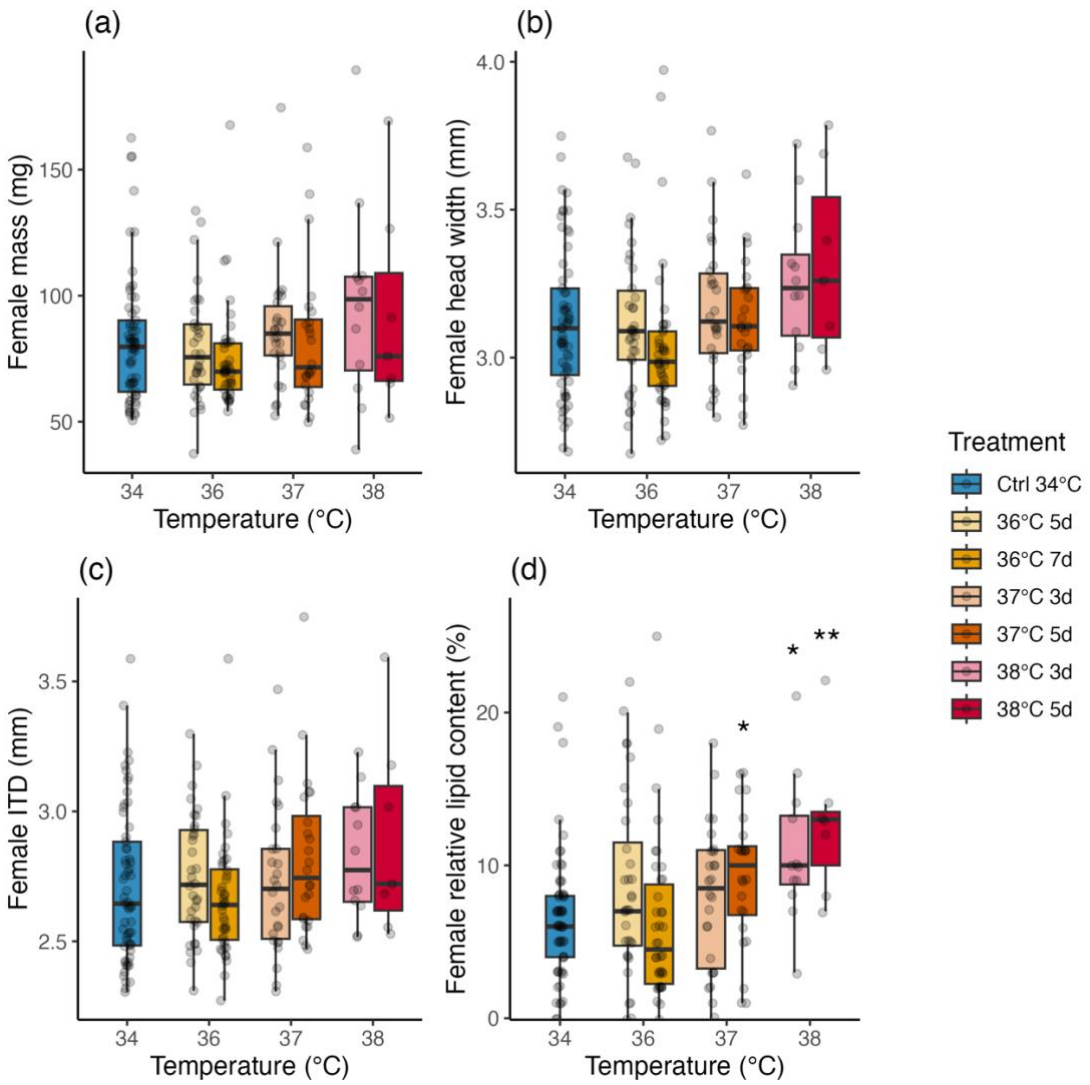

Figure 2. Effects of pupal heatwave exposure on adult female morphometrics and lipid content in *B. terrestris*: (a) adult mass, (b) head width, (c) intertegular distance (ITD), (d) and relative lipid content (2-day-old adults). Controls (34°C, blue) were compared to individuals exposed to increased temperatures: of 36°C for 5 days (light warm yellow) and 7 days (warm yellow), 37°C for 3 days (light reddish-orange) and 5 days (reddish orange), and 38°C for 3 days (light red) and 5 days (deep red). Asterisks denote significant differences (* $p < 0.05$, ** $p < 0.01$).

We found that heatwave intensity impacted wing development (GLMM: $\chi^2 = 9.992$, df = 1, $p < 0.01$, figure 3a). Specifically, 38C3d pupae were four times more likely to develop deformed wings compared to the controls ($z = -3.367$, $p < 0.01$), while 38C5d pupae were nearly seven times more likely of developing deformed wings ($z = -3.920$, $p < 0.001$). Pupae of the 36C7d treatment showed a marginally significant, twofold increase in deformed wings ($z = -2.482$, $p = 0.052$).

Our geometric morphometrics analysis without deformed wings indicated a significant effect on female wing shape (PERMANOVA: $F = 12.484$, $R^2 = 0.21$, $df = 5$, $p < 0.001$, figure 3b), but not on male wing shape ($F = 0.269$, $R^2 = 0.01$, $df = 2$, $p > 0.05$, figure S4b). Pairwise comparisons revealed significant differences between all treatment groups and the controls (36C5d, $F = 9.860$, $p < 0.05$; 36C7d, $F = 7.599$, $p < 0.05$; 37C3d, $F = 25.643$, $p < 0.05$; 37C5d, $F = 31.365$, $p < 0.05$; 38C3d, $F = 29.824$, $p < 0.05$). Similarly, we found that heatwave exposure affected centroid wing size in females (Kruskal-Wallis: $\chi^2 = 18.633$, $df = 5$, $p < 0.01$, figure S3c), but not in males ($\chi^2 = 0.657$, $df = 2$, $p > 0.05$, figure S4b). Pairwise comparisons indicated marginally significant decrease in wing size for females of the 36C7d treatment ($z = -2.279$, $p = 0.057$).

Furthermore, our heatwave treatment significantly affected the DA in female wings (LM: $F = 9.466$, $df = 5$, $p < 0.0001$, figure 3d), but not FA (Kruskal-Wallis: $\chi^2 = 3.903$, $df = 5$, $p > 0.05$, figure 3e). Females exposed to 36°C showed higher DA (36C5d, $t = -2.520$, $p < 0.05$; 36C7d, $t = -2.536$, $p < 0.05$), while females exposed to 37°C (37C3d, $t = 2.655$, $p < 0.05$; 37C5d, $t = 2.932$, $p < 0.05$) and 38°C (38C3d, $t = 2.500$, $p < 0.05$) showed lower DA compared to controls. In males, we found no effect on DA (Kruskal-Wallis: $\chi^2 = 1.318$, $df = 2$, $p > 0.05$, figure S4d), but on FA ($\chi^2 = 6.932$, $df = 2$, $p < 0.05$, figure S4e). Males of 37C3d ($z = -1.884$, $p < 0.05$) and 37C5d ($z = -2.547$, $p < 0.05$) showed slight increase in FA compared to controls.

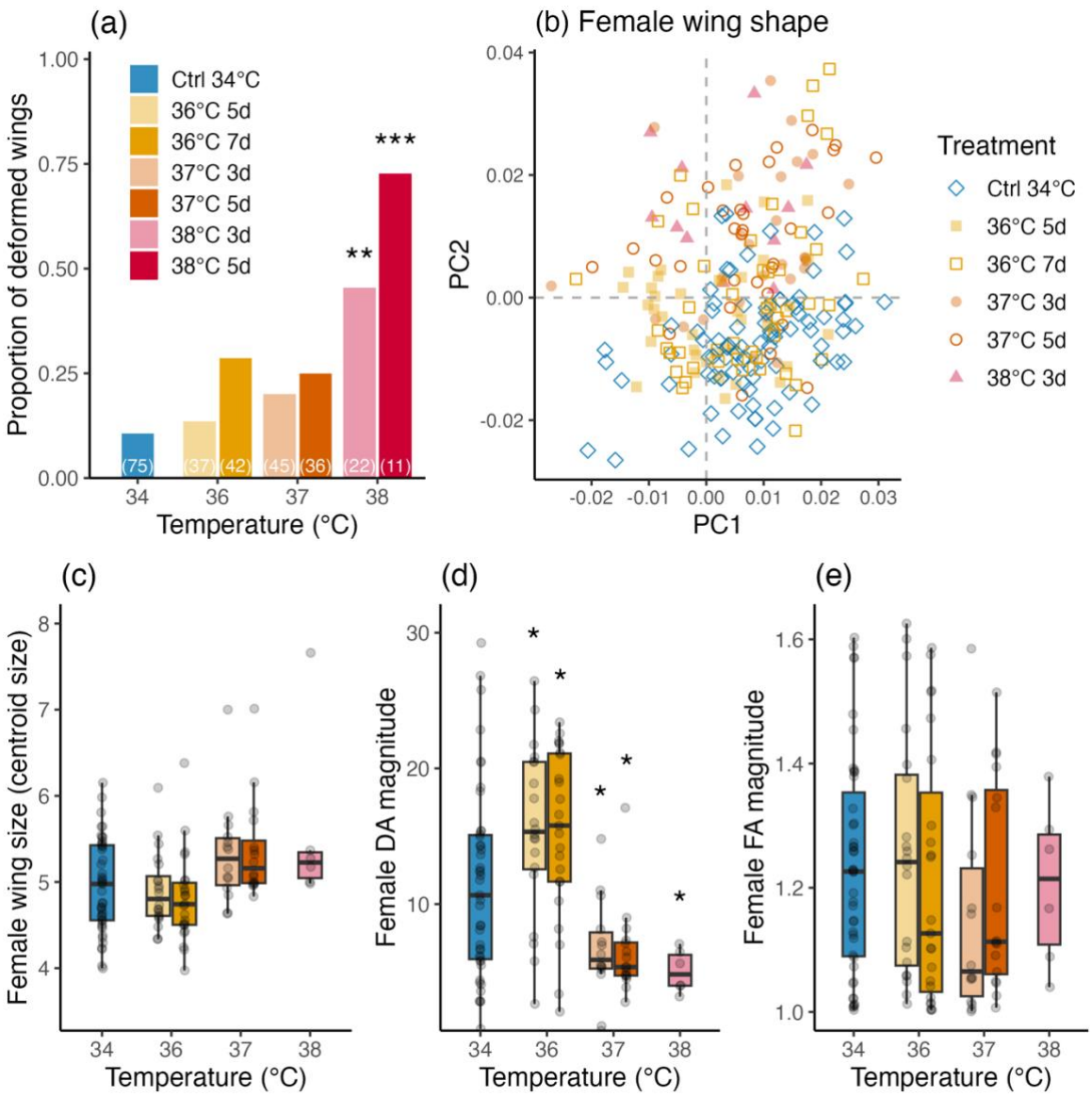

Figure 3. Heatwave effects on wing development in *B. terrestris*. (a) Proportion of deformed wings in emerged adult bees for each treatment group. (b) Principal component analysis (PCA) of wing morphology in emerged females with fully developed wings across heatwave treatments (see legend; 95% confidence intervals are shown in figure S4a). (c) Influence of heatwave treatments on wing size (centroid size) and the magnitude of (d) directional and (e) fluctuating asymmetry in fully developed female wings. Controls (34°C, blue) were compared to individuals exposed to increased temperatures: of 36°C for 5 days (light warm yellow) and 7 days (warm yellow), 37°C for 3 days (light reddish-orange) and 5 days (reddish orange), and 38°C for 3 days (light red). Asterisks denote significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4. Discussion

Our study provides compelling evidence that heatwave length and severity significantly impact pupal development in the key pollinator *B. terrestris* with notable effects on emergence rate (figure 1a), developmental duration (figure 1b), lipid stores (figure 2d), and wing development (figure 3). These findings highlight the extreme vulnerability of the pupal stage to heatwaves, particularly at 38°C, which appears to be a critical threshold for development disruption, aligning with our previous study on L4 larvae (Woegler & Kurze, 2024). Although our treatments are extreme, they reflect potential heatwave scenarios in Europe (Lhotka & Kyselý, 2024; Lhotka et al., 2018; Perkins-Kirkpatrick & Lewis, 2020; Simolo & Corti, 2025; Stillman, 2019). Recent heatwaves reaching or even exceeding temperatures of 38°C were, for example, reported in parts of the British Isles, Mediterranean and Eastern Europe (Barriopedro, Fischer, Luterbacher, Trigo, & García-Herrera, 2011; Lhotka & Kyselý, 2024; Rita et al., 2020).

Most notably, our findings show that emergence rates decreased by almost twofold and fivefold in pupae exposed to 38°C for 3 and 5 days, respectively, compared to controls (figure 1a). Despite *in vitro* rearing of L4 larvae and pupae deviates from natural nest conditions, the high emergence rate of controls suggests that our experimental setup allowed for normal development. While 38°C exposure slightly prolonged pupal development, a 36°C exposure slightly accelerated it (figure 1b), suggesting a potential developmental benefit in pupae to temperature elevation up to 37°C, followed by severe disruption at 38°C. Given the relative heat tolerance of our model species, *B. terrestris*, we expect cold-adapted bumblebee species such as *B. lapidarius*, *B. alpinus*, and *B. poralris* to be even more vulnerable to heatwaves (Martinet et al., 2021; Zambra, Martinet, Brasero, Michez, & Rasmont, 2020). Therefore, extreme heatwave events could have a devastating impact on bumblebee populations, which could lead to local extinctions and a decline in species diversity, with significant implications for crop pollination and ecosystem services (Ghisbain et al., 2024).

We observed no clear heatwave effects on sex-specific body size proxies (figures 2 and S3), except for an increased female head width at 38°C. These results contrast with previous studies showing that colonies exposed to prolonged heat stress would produce smaller offspring, indicated by smaller ITD (Guiraud et al., 2021; Gérard et al., 2023) and body mass (Perl et al., 2022), potentially reflecting resource allocation strategies such as reduced larval feeding. Alternatively, heat stressed larvae may have a decreased body weight gain due to reduced food consumption (Woegler & Kurze, 2024). We speculate that our morphometric data potentially reflect a selective advantage of larger pupae under heat stress. Although small insect body sizes should generally be favoured under warmer environmental conditions over the long term (González-Tokman et al., 2020), aligning with Bergmann's rule, larger workers in numerous ant species have been described to be more heat resistant than smaller workers (Perez & Aron, 2020). In addition, we found increased relative lipid content (fat body) in females with increasing heatwave intensity (figure 2d), indicating a potentially higher heat stress resilience due to increased energy reserves, immunocompetence, and heat shock protein (HSP) synthesis (Arrese & Soulages, 2010; King & MacRae, 2015; Korner & Schmid-Hempel, 2004; Vanderplanck et al., 2021; Vesterlund, Lilley, Van Ooik, & Sorvari, 2014). Interestingly, the relative lipid content was not affected in heat stressed L4 larvae, further underscoring the differential vulnerability of developmental stages (Woegler & Kurze, 2024).

Wing development was severely impaired by heatwave intensity, notably with a fourfold and sevenfold increase in wing deformities in emerged adult bees from pupae exposed to 38°C for 3 and 5 days, respectively (figure 3a). Even excluding individuals with these deformities, females wing shape altered for all treatments (figure 3b), which appears to be a natural response in colonies under heat and parasitic stress (Gérard, Cariou, et al., 2022; Gérard et al., 2018). However, our data shows only marginal effects on wing size (figure 3c) with a similar pattern as for the other body size proxies (figure 2). DA was affected by temperature in females, with increasing DA at 36°C and decreasing DA at 37°C and 38°C (figure 3d), potentially also reflecting the different selection pressures among treatments. While FA remained unaffected in females (figure 3e), confirming

previous studies in honeybees (Jones, Helliwell, Beekman, Maleszka, & Oldroyd, 2005) and bumblebees (Gérard et al., 2018), males showed a slight increase in FA at 37°C (figure S4e).

In conclusion, our study provides novel insights into the direct impacts of varying heatwaves scenarios on *B. terrestris* pupal development, eliminating confounding colony-level effects. While our experimental design sacrifices some ecological realism, it enabled precise control over timing and temperature to match potential heatwave scenarios, making our study relevant for predicting future pollinator declines. Our findings highlight a critical heat stress threshold at 38°C, where severe disruptions in bumblebee pupal development manifest by particularly impairing emergence and wing development. Given the heat tolerance of *B. terrestris*, we expect other cold-adapted bumblebee species to be even more vulnerable to increasingly more frequent and severe heatwaves as climate change continues to cause more extreme weather events. Therefore, our findings underscore the profound fitness implications for bumblebee colonies with cascading consequences for whole populations, biodiversity, crop pollination and ecosystem services of bumblebees as key pollinators.

Data accessibility

All data and statistical code output supporting the findings of this study are provided in the electronic supplementary material.

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Author's contributions

SL and CK collected the data. CK conceived, designed, and supervised the study; analysed and interpreted the data; and wrote the manuscript. SL gave approval for publication.

Ethics statement

This study was conducted in accordance with the ethical regulations of the German Animal Welfare Act (TierSchG) for conducting experiments with insects.

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Competing interests

The authors have no competing interests.

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Supplementary information

Heatwave length and severity drive development disruption in a key pollinator

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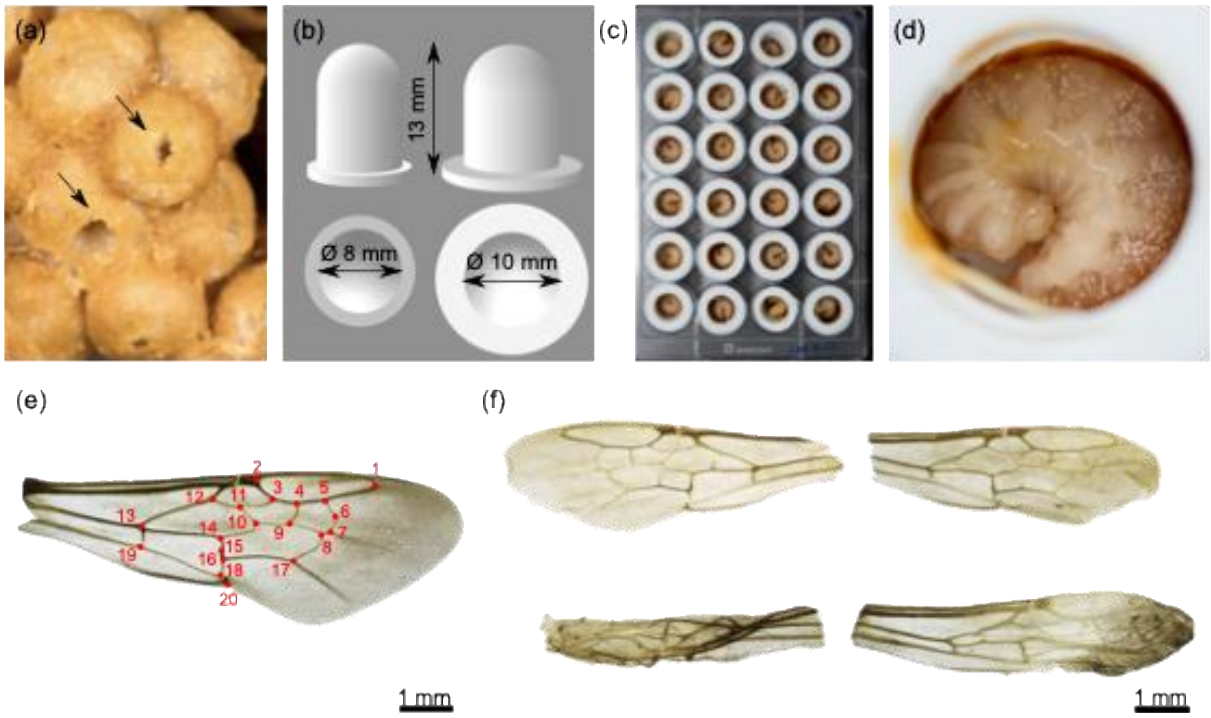

Figure S1. *In vitro* larval rearing of L4 (a-d) and wing morphology analysis (e, f). (a) L4 larvae were identified by the small opening for food provisioning (black arrows) and carefully collected. (b) Design of 3D-printed polylactide (PLA) artificial brood cells (capacity: 0.6 ml or 0.96 ml). (c) L4 larvae inside artificial brood cells in a 24-well plate. (d) Observation of L4 feeding on medium (see also supplementary video 1). (e) Fully developed right forewing showing the positions of the 20 landmarks (red dots) used for the geometric morphometric analysis, including centroid size, wing shape, directional asymmetry (DA), and fluctuating asymmetry (FA). (f) Representative examples of left and right forewings from two emerged adults with slight (top) and more severe (bottom) wing deformities. Individuals with deformed wings were excluded from further geometric morphometric analyses. Scale bar = 1mm.

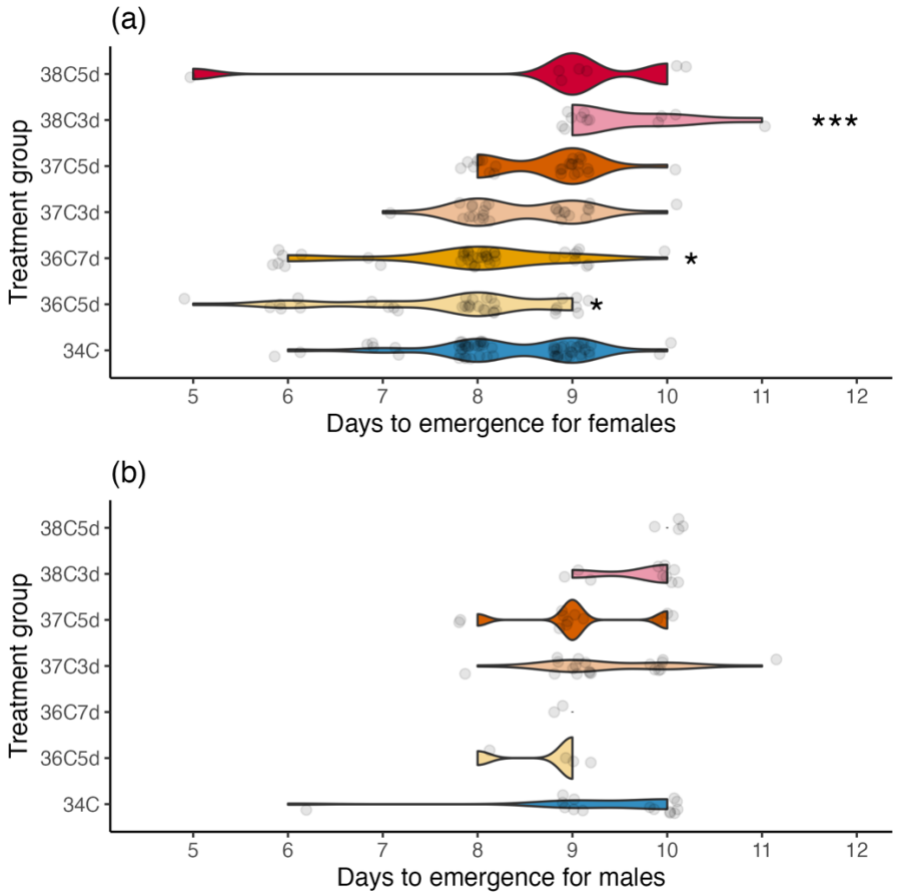

Figure S2. Direct effects of heatwaves of varying intensity on (a) female and (b) male developmental duration in *B. terrestris* pupae. Controls (Ctrl, reared at constant 34°C, blue) were compared to individuals exposed to increased temperatures: of 36°C for 5 days (light warm yellow) and 7 days (warm yellow), 37°C for 3 days (light reddish-orange) and 5 days (reddish orange), and 38°C for 3 days (light red) and 5 days (deep red). Significant differences are marked by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

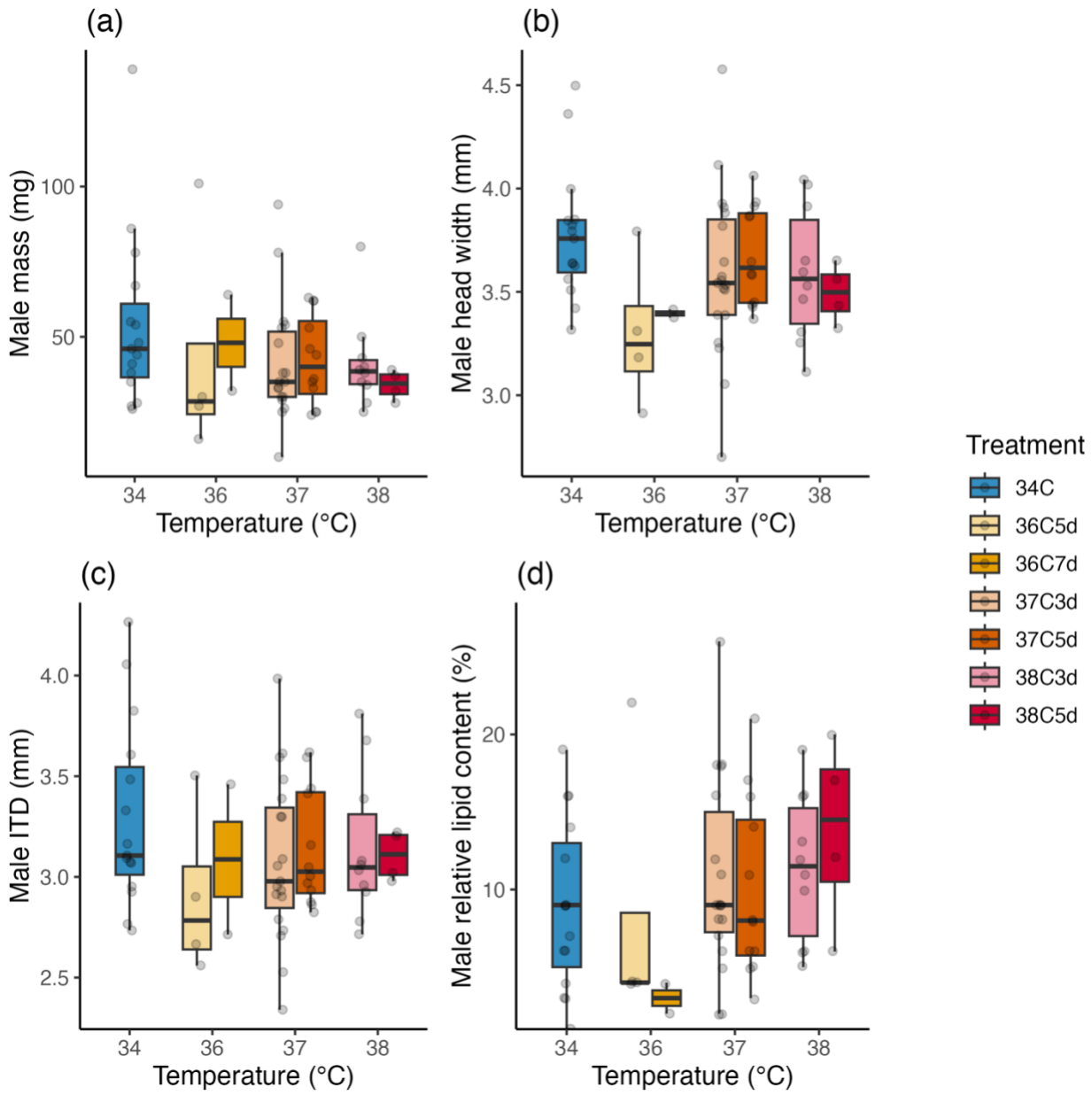

Figure S3. Effects of heatwaves intensity and duration during *B. terrestris* pupal development on adult male morphometrics and lipid content: (a) adult mass, (b) head width, (c) intertegular distance (ITD), (d) and relative lipid content (2-day-old adults). Controls (34°C, blue) were compared to individuals exposed to increased temperatures: of 36°C for 5 days (light warm yellow) and 7 days (warm yellow), 37°C for 3 days (light reddish-orange) and 5 days (reddish orange), and 38°C for 3 days (light red) and 5 days (deep red). Asterisks denote significant differences (* $p < 0.05$, ** $p < 0.01$).

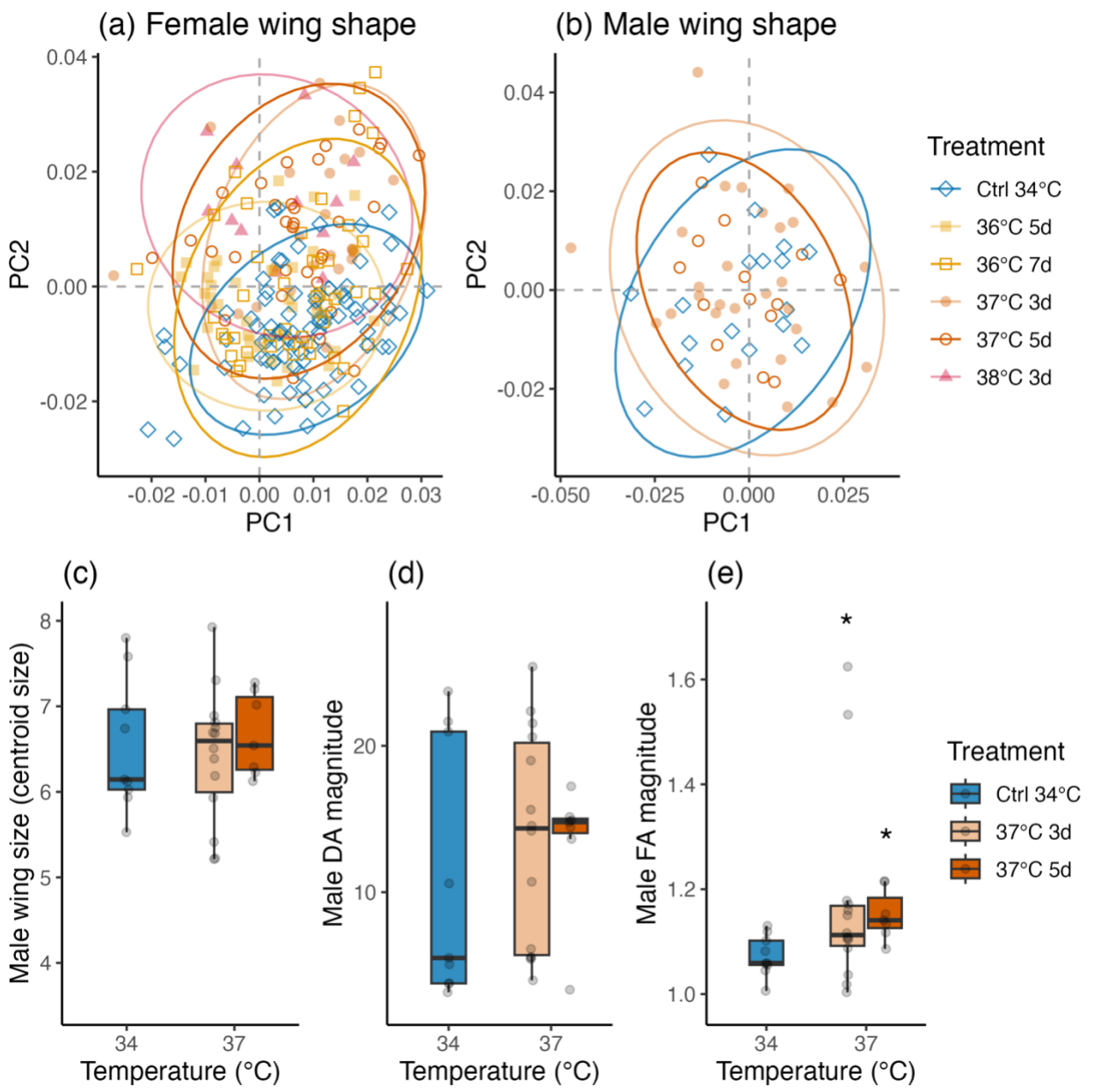

Figure S4. Heatwave effects on wing development in male *B. terrestris*. (a, b) General wing morphology variation visualised by principal component analysis (PCA) for females (a) and males (b) with fully developed wings across heatwave treatments (see legend). 95% confidence intervals are shown as ellipses. (c) Influence of heatwave treatments on wing size (centroid size) and the magnitude of (d) directional (DA) and (e) fluctuating asymmetry (FA) in fully developed male wings. Controls (34°C, blue) were compared to individuals exposed to increased temperatures of 37°C for 3 days (light reddish-orange) and 5 days (reddish orange). Asterisks denote significant differences (* $p < 0.05$).