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Impacts of high temperature during early capped brood on pupal development and the size of appendages in adult workers *Apis cerana*

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Whether the development of honeybee broods is healthy or not determines the productivity of bee colonies. Pupation is a critical period in the development of holometabolous insects, characterized by the transition from larva to pupa, and its sensitivity to high temperature was investigated in *Apis cerana* worker bees. Mature larvae (ML), the first and second days of prepupa (PP1 and PP2), and the first day of pupa (P1) were exposed to 40°C for varied durations of time. The mortality, development duration, birth weight, size of the body, and appendages of eclosed *Apis cerana* worker bees were measured. Results showed that PP1 had the highest mortality, the lowest birth weight, and the longest development duration among the pupation stages. When exposed to 40°C for 12 h and 16 h, more than 28 and 84% of PP1 failed to complete development, respectively. Additionally, high-temperature treatment had a significant effect on the length of the proboscis, the size of the forewing, and the size of the hind leg. These findings suggest that ML and PP1 are crucial checkpoints for wing and appendage (proboscis and legs) development and provide insights into the mechanisms of honeybee brood susceptibility to high-temperature stress in the context of global warming.

KEYWORDS

high-temperature stress, pupation, honey bee, appendage, development

1. Introduction

Honeybees are incredibly proficient at transferring pollen and provide essential pollination services for both cultivated and wild plants globally. However, their exposure to abiotic and biotic stressors has caused severe health issues in honeybees, which has received much attention in the context of global warming (Le Conte and Navajas, 2008; Rader et al., 2013; Abou-Shaara et al., 2017). Temperature is one of the abiotic factors influencing the growth and development of honeybees and other ectotherms (Sun et al., 2021).

Although honeybee colonies have adapted to a wide range of temperatures, their normal development relies on the nest temperature being regulated by the mass of adults (Kronenberg and Heller, 1982; Heinrich, 1993). The temperature of the brood area in the nest is typically maintained close to a constant (35°C), indicating that honeybee brood is sensitive to temperature fluctuations (Kleinhenz et al., 2003; Stabentheiner et al., 2010; Zhou et al., 2015). Heat waves or artificial mite-killing hyperthermia in colonies can have lethal consequences for immature honeybees, even if only exposed to high temperatures for a short period (Medina et al., 2018; Kablau et al., 2020).

Honeybee broods are susceptible to thermal stress. The capped period (last instar, prepupa, and pupa) rearing at suboptimal temperatures has been shown to have detrimental effects on adult honeybees. Studies have demonstrated that such thermal stress during the capped period can impair mushroom body development, learning and memory abilities (Tautz et al., 2003; Groh et al., 2004; Jones et al., 2005), behaviors (Becher et al., 2009), and wing morphology (Tan et al., 2005; Szentgyörgyi et al., 2018) in the resultant adult bees. Short-term heat waves of 40°C or higher have been linked to increased fluctuating asymmetry in forewing shape or abnormal venation reduced longevity and decreased age at the onset of foraging, body size and sucrose responsiveness, and male fertility in the resultant adult bees (Medina et al., 2018; Zhu et al., 2018; Kablau et al., 2020). Additionally, suboptimal brood-rearing temperatures have been found to reduce the adult bees' ability to resist external environmental pressures and disease (Flores et al., 1996; McMullan and Brown, 2005; Medrzycki et al., 2010).

The internal structures of holometabolous insects experience drastic changes during the larva-pupa transition, implying that the prepupae are more susceptible to stress than other developmental stages (Wang et al., 2016; Kablau et al., 2020). To ascertain the sensitivity of the early capped broods of indigenous *Apis cerana* to high temperatures that undergo metamorphosis and how heat exposure affects their developmental characteristics and morphology traits, these broods were exposed to a high temperature of 40°C for different periods of time. In this study, we answer the following questions: (1) Which stage, from mature larva to new pupa, is more sensitive to high temperature? (2) To what extent does high temperature affect the development process and morphological traits?

2. Materials and methods

2.1. Honeybee brood and high-temperature treatment

An experiment was conducted in the spring season in Fuzhou, Southeast China, with three healthy colonies of *Apis cerana* with strong populations. To ensure the eggs were of the same age, each queen was confined in a mesh cage to lay eggs on a new comb. After 8.5 days, the larvae were monitored until they were capped within 4 h, after which they were cut out with a comb patch and transferred into the incubators immediately (recorded as start time, 35°C ± 0.2°C, relative humidity 75 ± 5%). The control group was maintained at 35°C ± 0.2°C until eclosion. The comb patches incubated for 1 day (mature larvae, ML), 2 days (prepupa, PP1), 3 days (prepupa, PP2), and 4 days (pupa, P1) were transferred to high temperature (40°C ± 0.2°C) for 16 h before being returned to 35°C until eclosion. The survival rates were recorded to determine which stage was more vulnerable to high temperatures.

After identifying the sensitive stage, the heat time effect on its development parameters was further investigated. The honeybees in the PP1 stage, being the most sensitive, were exposed to 40°C for 8 h, 12 h, and 16 h, before it was recovered to 35°C until emergence. The newly capped broods reared at 35°C until emergence were used as the control group. A triplicate of newly capped brood patches (>22 capped brood) from three colonies per treatment or control group simultaneously were harvested to reduce the influence of factors such as heredity and nutrition on the results.

2.2. Mortality, birth weight, and development duration measurement

Upon the emergence of the capped brood, the comb was inspected every hour, and the number of eclosed bees was recorded as the birth weight. An electronic balance (±0.1 mg, Sartorius, Germany) was used to weigh each bee immediately. If the capped cells did not emerge within 15 days post-capping, they were deemed dead (which is 3 days longer than the usual eclosion period). The development duration of each honeybee since the start time was also recorded. All treatment groups and control groups were repeated with three colonies.

2.3. Morphology measurement

Dissections and mounting of the proboscis, right forewing, and the right hind leg of eclosed bees (20 bees in each colony, 3 colonies per group, except for the 20 workers analyzed in PP1 that had high mortality upon eclosion) onto slides under a stereoscope were conducted. Subsequently, six appendage characteristics, including proboscis length, forewing length, forewing width, femur length, tibia length, and metatarsus length of each bee, were measured according to the methods outlined by Ruttner (1988) and Zhu et al. (2017).

2.4. Statistical analysis

Mortality data between treated groups were examined using one-way ANOVA after the transformation of arcsin (square root) of the mortality rates (SNK, Wang et al., 2016). Additionally, a principal component analysis (PCA) was conducted to determine parameters of overall body sizes with 17 morphological traits measured on individual bees. The correlation matrix procedure was selected as the resulting principal component 1 (PC1) was found to be more closely related to body size (Poot-Báez et al., 2019). The coefficients for each PC were then used to calculate scores as individual measures of appendage sizes. The PCA was completed using the R packages FactoMineR (Lê et al., 2008). A one-way ANOVA was also used to compare the variance of the development

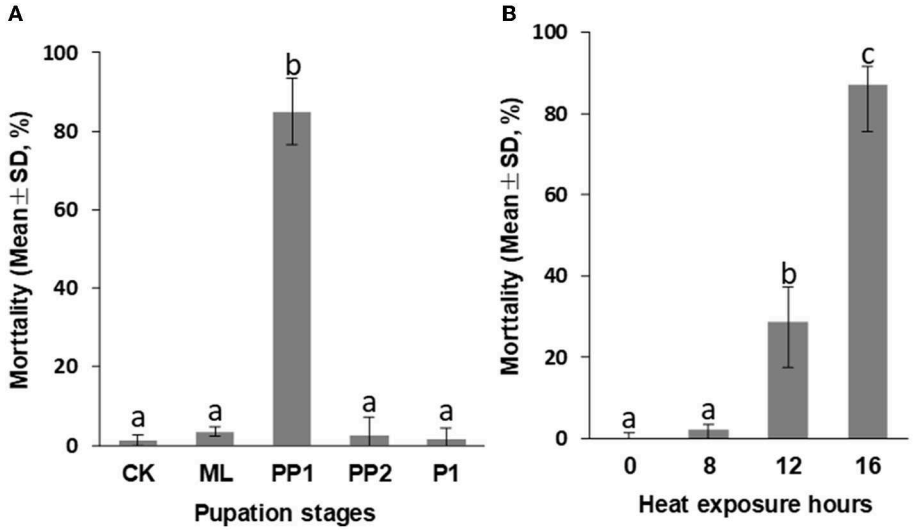

FIGURE 1
Mortality of honeybee broods exposed to 40°C for 16 h **(A)** and PP1 exposed to 40°C for 0 (CK), 8, 12, 16 h **(B)**. CK, control group; ML, mature larva; PP1, the first day of prepupa; PP2, the second day of prepupa; P1, the first day of the pupa. The different letters in the lowercase indicate a significant difference at the $P < 0.01$ level.

duration, birth weight, and morphological characteristics between treatments, with a *post-hoc* analysis conducted using the Student–Newman-Keuls tests (SPSS 21.0). Data were represented as means ± standard deviations.

3. Results

3.1. Mortality, birth weight, and development duration

The early prepupal honeybee (PP1) was found to be the most sensitive to high-temperature stress among the test groups during the larva-pupa transition. When exposed to 16 h at 40°C, the PP1 group showed an average mortality rate of 84.94%±8.49%, significantly higher ($P < 0.01$, Figure 1A) than the control, ML, PP2, and P1 groups, which had < 5% mortality and no difference between them ($P > 0.05$, Figure 1A).

In comparison with the control group (92.38 ± 9.19 mg), ML (88.96 ± 5.87 mg), PP2 (88.72 ± 6.10 mg), and P1 (89.08 ± 7.01 mg) groups, the PP1 group (84.30 ± 4.46 mg) significantly reduced birth weight and increased development duration ($P < 0.01$, Figure 2). The ML, PP2, and P1 groups showed no difference in birth weight with the control group ($P > 0.05$), but the PP1 group decreased its birth weight significantly ($P < 0.01$) under the same condition. The development durations in the ML, PP1, and P1 groups, excluding PP2, increased significantly ($P < 0.01$, Figure 1B) compared to the control group. These results suggest that the PP1 stage is the most vulnerable to thermal stress, which delays the rate of honeybee pupation and post-stress development.

Furthermore, we observed a significant increase in PP1 mortality rates as heat exposure time was prolonged. An increase in heat exposure from 8 h to 16 h resulted in a mortality rate increase from 2.17 to 87.08% ($P < 0.01$, Figure 1B), a decrease in birth weight from 95.40 mg (8 h exposure) to 84.30 mg (16 h exposure; $P < 0.01$, Figure 3A), and an extension of development duration from 11.48 days to 11.71 days, significantly ($P < 0.01$; Figure 3B). Additionally, an 8-h exposure to 40°C did not increase mortality or birth weight but did increase the development duration compared to the control group, suggesting an 8-h heat exposure may cause subtle damage to the honeybee development.

3.2. Wing and appendage size

Exposure to high temperatures for 16 h significantly reduced the proboscis length of eclosed bees in the ML, PP1, and PP2 groups ($P < 0.01$, Figure 4) compared to the CK group. The PP1 bees had the shortest proboscis length, followed by the ML and PP2 groups. However, no significant decrease was observed in the P1 group ($P > 0.05$; Supplementary Table 5). The forewing size decreased significantly in all treatment groups, with the ML group showing the smallest wing length and width, followed by the PP2, PP1, and P1 groups ($P < 0.01$, Figure 4). High-temperature stress on prepupal stages (PP1 and PP2) had the most significant impact on the hind leg size ($P < 0.01$, Figure 4), followed by the ML group, while there was no impact on the hind leg size of the P1 group in terms of the femur and metatarsus length ($P > 0.05$, Figure 4).

After 8, 12, and 16 h of high-temperature treatment during the PP1 period, a significant decrease in proboscis length ($P < 0.01$), forewing width ($P < 0.01$), femur length ($P < 0.01$), tibia length ($P < 0.01$), metatarsus length ($P < 0.01$), and metatarsus width was observed ($P < 0.01$, see the Supplementary material). As heat exposure time prolonged, morphological characteristics were further diminished, implying that the heat impairment

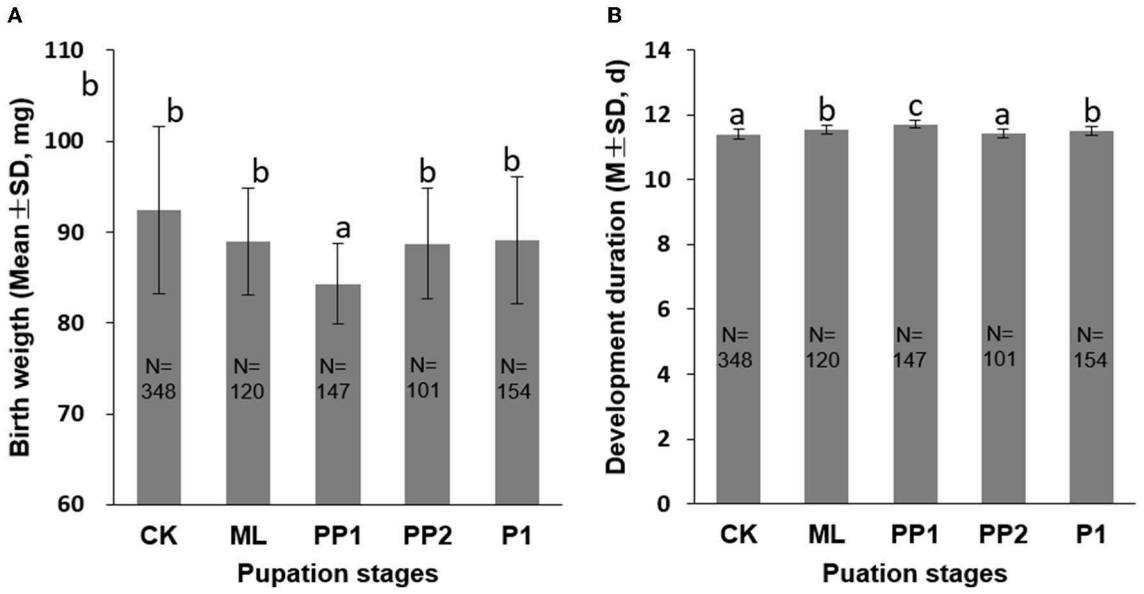

FIGURE 2
Birth weight **(A)** and development duration **(B)** of honeybee broods exposed to 40°C for 16 h. CK, control group; ML, mature larva; PP1, the first day of prepupa; PP2, the second day of prepupa; P1, the first day of the pupa. The different letters in the lowercase indicate a significant difference at the $P < 0.01$ level. The numbers in the columns indicate sample sizes.

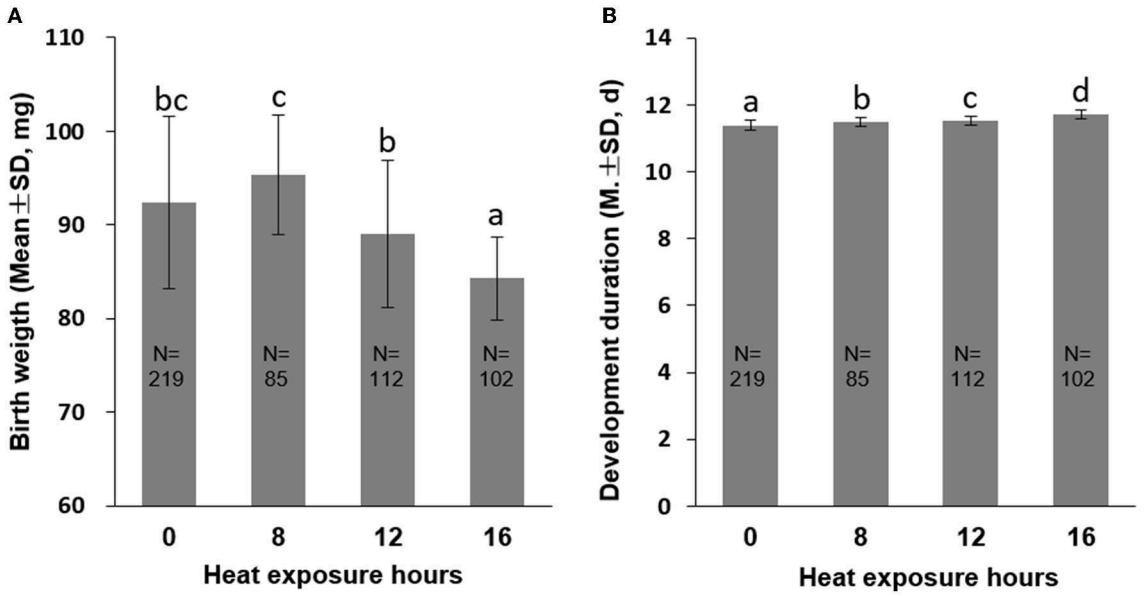

FIGURE 3
Birth weight **(A)** and development duration **(B)** of honeybee brood PP1 exposed to 40°C for 0 (CK), 8, 12, and 16 h. The different letters in lowercase indicate a significant difference at the $P < 0.01$ level.

had a cumulative effect on the metamorphic staged brood honeybees.

The results of the PCA analysis indicated that the test groups exposed to high-temperature stress for 16 h were morphologically different from the CK group. Notably, the PP1 group, followed by the ML and PP2 groups, was grouped distinct from the CK group, indicating that PP1, among the pupation stages, was particularly susceptible to deviated high-temperature stress (Figure 5).

4. Discussion

The pupal stage of holometabolous insects affords them greater resistance to hostile environmental conditions, yet the pupation process from larva to the pupa is a fragile stage that is sensitive to the environment. Hironaka and Morishita (2017) proposed that holometabolous insects evolved pupal development stages to tolerate harsh environments in both winter and summer. Our

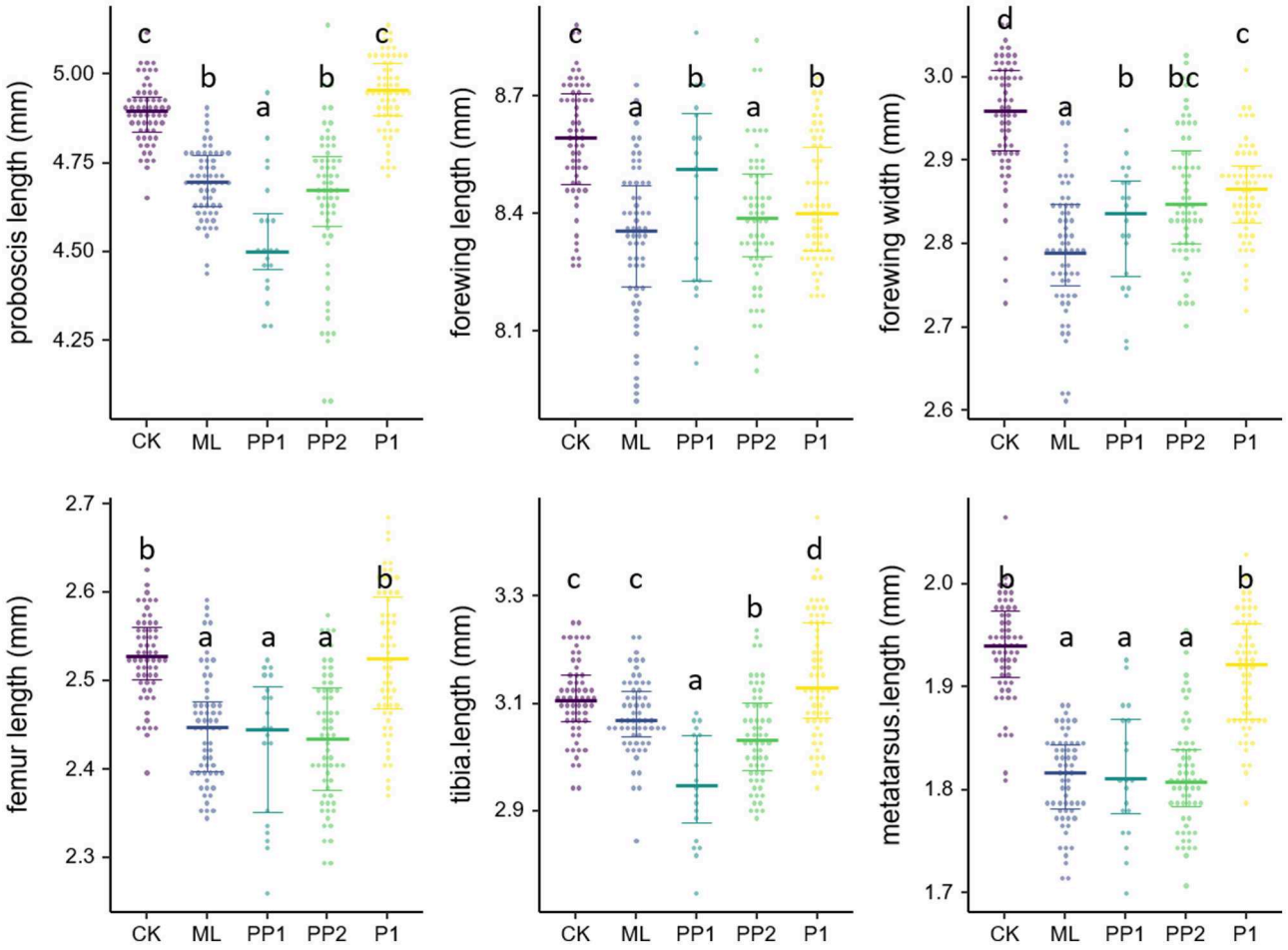

FIGURE 4
Wing size and appendage size of honeybee broods exposed to 40°C for 16 h. Colored dots represent experiment groups: CK, control group. ML, mature larva; PP1, the first day of prepupa; PP2, the second day of prepupa; P1, the first day of the pupa. Crossbars with whiskers in each group indicate the median value and the 25th and 75th percentiles.

study examined the effects of high temperatures on the mortality rate, development duration, and birth weight of honeybees during the transition from the cessation of feeding at the mature larval stage to the molting into pupae. It was determined that the early pupae PP1 stage was the most sensitive to high-temperature stress among the metamorphic stages, akin to our previous finding that 3-day-old capped broods were susceptible to low temperature (Wang et al., 2016). During the metamorphic period, their internal tissues and organs undergo tremendous changes (Myser, 1954). Moreover, several studies have demonstrated that pupation is particularly vulnerable to abnormal temperatures and nest spleen shaking (Jay, 1963; Bennett et al., 2015; Zhu et al., 2018). To further assess the effects of high temperatures on early capped brood development and the size of appendages (wings, legs, and proboscis) in eclosed honeybees, we examined the response to thermal stress at different stages from mature larvae to pupa. Based on the extent of the response to a high temperature between stages, our findings indicated that the early pupae PP1 stage was the most sensitive among the metamorphic stages. This stage-specific response to thermal stress can help to better understand the mechanisms underlying the thermal stress on honeybee development, as well as to assess the potential damage to honeybee development in heat, such as under global warming and heat waves (Le Conte and Navajas, 2008; Rader et al., 2013) and heat treatment in hive mite control (Kablau et al., 2020). To preserve or cultivate bee pupae effectively, it is essential to maintain an accurate and stable development temperature during this delicate period, or else the quality of the emerging bees may be impaired. Simultaneously, there could be a vulnerable stage of development for damaging insects. This study also proposes a method of controlling these insects.

High temperatures can have a detrimental effect on the hormone regulation system, an essential component of bee growth and development. This study revealed that exposing pupae to a temperature of 40°C for 16 h caused PP1 with marked pupal mortality and delayed development durations. This could be attributed to the disruption of hormones responsible for the pupation process, such as juvenile hormones and ecdysone. The metamorphosis and development of insects are regulated

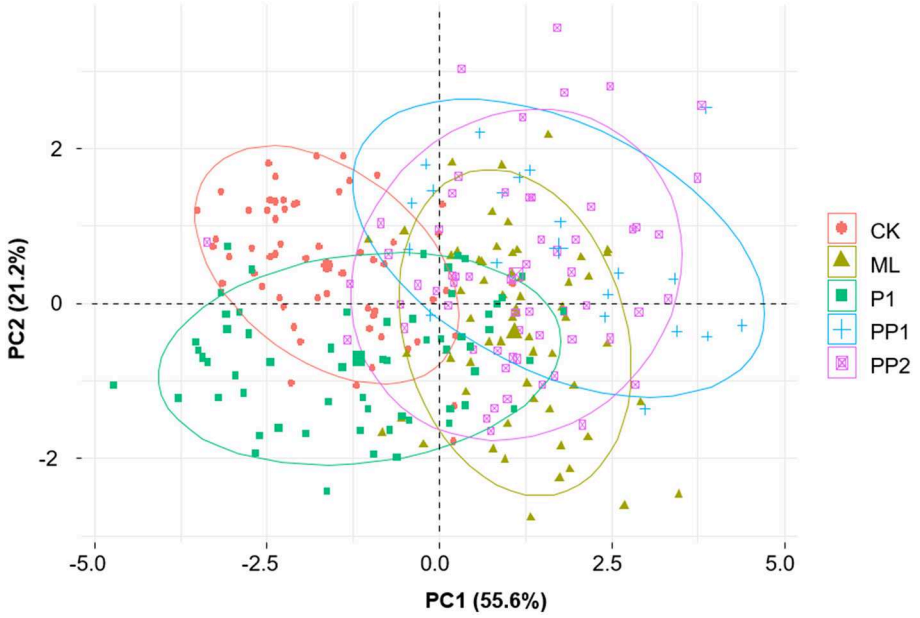

FIGURE 5
PCA plot for the appendage sizes in honeybee broods exposed to high temperatures for 16 h. Each dot indicates the appendage sizes of an individual bee, and the shapes of colored dots represent experiment groups: CK, control group; ML, mature larva; PP1, the first day of prepupa; PP2, the second day of prepupa; P1, the first day of the pupa. The horizontal and vertical axes represent PC1 and PC2, respectively. The numbers in the parentheses show the proportion of the variance explained by PC1 and PC2. Morphologically similar samples will be plotted close to each other (ellipse of 90% confidence interval). Raw data are available in Supplementary Table 5.

by juvenile hormones and ecdysone (Thummel, 2001). Cold temperatures were shown to increase the juvenile hormone titers in nurses and foragers (Lin et al., 2004), indicating its potential as a stress hormone. JH antagonizes ecdysone in metamorphic transition, which may inhibit the development process (Lyu et al., 2019). A rise in JH titer in response to high temperatures may delay the transition from prepupa to pupa, thus prolonging the development duration (Supplementary Table 2). Additionally, we reported that a key transcription factor *FoxO*, regulated by the insulin signaling pathway, has been identified as being involved in suppressing the pupation process (Yao et al., 2020). Despite the knowledge gained from current research, the physiological, and molecular mechanisms behind the thermal damage in honeybee metamorphic stages have yet to be determined. Further investigations are needed to elucidate the effects of high temperatures on the degradation of juvenile hormones, the synthesis of ecdysone, and hormone-regulated signal transmission.

Our findings indicated that thermal stress during metamorphic stages had an effect on tissue remodeling and morphogenesis during pupation, leading to a decrease in the size of the proboscis, legs, and wings in adults. Notably, the most pronounced effect was seen on forewing size during the mature larvae stage. It was consistent with our previous study that abnormal veins mainly occurred at ML under low temperatures, with the highest abnormal rate and the largest variety of types (Zhu et al., 2018). These findings indicate that the ML stage is probably a checkpoint for wing development in *Apis cerana* workers and other holometabolous insects (Nardi and Norby, 1987). Additionally, our data showed that the prepupal stages (PP1 and PP2) were particularly vulnerable to thermal stress with regard to the proboscis and leg development. However, the development of tergites, wax plates, and sternites was not affected by high temperatures. Collectively, the development of wings and ventral appendages (proboscis and legs), which are vulnerable to thermal stress in early prepupae, indicates that the early capped brood honeybee had a stage-specific response to thermal stress. The productivity of adult bees is closely connected to the development of honeybee appendages. Therefore, it is of great importance to explore the molecular mechanism of how high temperature influences appendage development.

5. Conclusion

Our experiments with mature larvae, first- and second-day pupae, and new pupae *Apis cerana* exposed to high temperatures demonstrated that heat exposure impairs honeybee development health, as well as wing, proboscis, and leg sizes. Moreover, the first-day pupae were found to be more susceptible to high-temperature stress, and mature larvae and the first-day pupae were essential stages for wing and appendage development in honeybees.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

Conceived and designed the experiments: XX, XD, SZ, BZ, and XZhu. Performed the experiments: XX, KL, QW, HL, CZ, XZha, and XZhu. Analyzed the data: XX, CZ, MC, and XZhu. Contributed to reagents, materials, and analysis tools: QW, XX, XZhu, and BZ. Wrote the paper: XX and XZhu. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer YF declared a shared affiliation with the author XD to the handling editor at the time of the review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1144216/full#supplementary-material

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