High temperature sensitivity of bumblebee castes and the colony-level costs of thermoregulation in *Bombus impatiens*

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Highlights:

- Larval CTmax is significantly lower than the CTmax of adult castes.
- Whole-colony thermoregulation is energetically costly at high and low temperatures.
- Colonies experiencing 30°C and below successfully thermoregulate nest temperature.
- Above 30°C, nest temperature cannot be regulated via colonial thermoregulation.

Abstract

Physiological thermal limits often reflect species distribution, but the role that ambient temperature (T_a) plays in limiting species within their thermal environment remains unclear. Climate change-linked declines in bumblebees, an important pollinator group, leave questions regarding which aspect of their physiology is hindered under high T_a. As a eusocial species, bumblebees utilize their ability to thermoregulate as a superorganism to maintain nest temperature (T_n) within a narrow thermal window to buffer developing larvae from developmental defects. Thermoregulatory behaviours, such as thermogenesis to warm up and fanning to cool down the nest, are energetically expensive and it is uncertain how successful large colonies are at maintaining T_n within its optimal range. Using a common bumblebee species, *Bombus impatiens*, our study first established the critical thermal limits (CTmax) of workers, queens, drones and larvae to determine which caste is most thermally sensitive to heat. We found that larvae had significantly lower heat tolerance than adults, highlighting the importance of colonial thermoregulation. We then measured the energy expenditure of large colonies under acute thermal stress (5-40˚C) using flow-through respirometry while simultaneously quantifying T_n. Colonies that experienced T_a at or below optimal T_n (≤30˚C) were successful at thermoregulation. At 35˚C and above, however, T_n increased despite high energetic costs to the colony. Together our results demonstrate that high T_a poses a risk to colonies that fail to buffer thermally sensitive larvae from changes in T_n.

Keywords

Heat tolerance, bumblebee, castes, thermoregulation, colony, *Bombus*, metabolic rate

1. Introduction

The capacity to function in variable environmental temperatures is central to animal species success and distribution. Macrophysiological patterns emerging to date indicate that measures of thermal tolerance are linked with species distribution, though this association is not straightforward. Terrestrial cold tolerance has greater latitudinal and altitudinal variation than upper thermal tolerance both on a broad scale across taxon groups (Sunday et al., 2019, 2014, 2011) and within taxa including lizards (e.g., Clusella-Trullas et al., 2011; Huey et al., 2009; Van Berkum, 1988), amphibians (e.g., Snyder and Weathers 1975) and insects (e.g., Addo-Bediako et al. 2000, Calosi et al. 2010, Oyen et al. 2016). Other factors than thermal tolerance may be better predictors of species distribution including foraging activity thermal limits (e.g., Guo et al. 2020), growing degree day and precipitation (e.g., Tremblay et al. 2021) as well as moisture and predator-prey interactions (e.g., Amundrud and Srivastava 2020). Nevertheless, at the population-level, upper thermal tolerance may respond to local climate and habitat (Herrando-Pérez et al., 2020; Vorhees et al., 2013). Recent findings suggesting that the ability to cope with temperatures that exceed species' historical upper thermal limits explained the decline of endothermic poikilothermic bumblebees (Soroye et al., 2020) and shrinking distributions at the southern ranges of this group of bees (Kerr et al., 2015). Whether physiological properties limit their capacity to cope with environmental temperature variation remains unclear.

The ability to cope with temperature variation is not easily characterized in animals such as eusocial bumblebees given their multifaceted thermal physiology. An individual's capacity to tolerate acute changes in environmental temperature can be characterized by measuring their critical thermal maxima (CTmax), a point of failure when locomotion is no longer possible (Berrigan and Hoffmann 1998). Bumblebee species show variation in CTmax according to altitude (Oyen et al., 2016) as well as sociality and nesting strategy, for example cavity-nesting bumblebees are less thermally tolerant than other ground-nesting bee species (Hamblin et al., 2017). Average estimates of CTmax values obtained for a species are fairly robust with no changes found according to acclimation temperature, feeding status, adult age (Oyen and Dillon, 2018) or caste (workers vs. queens; Maebe et al. 2021). Bumblebee colonies also include male caste as well as developing larvae and pupae, which may represent thermally vulnerable colony members. The "weak link" hypothesis discusses how individuals with the lowest CTmax are more easily thermally stressed and thus impact colony performance (Baudier and O'Donnell, 2017). Therefore, bumblebee colonies with diverse phenotypes, and possibly variable abilities to cope with heat stress, should be considered.

The endothermic poikilothermic capacity of bumblebees provides multiple benefits for individuals and the colony. Individuals can warm up and maintain flight muscle temperature above that of the environment, making them particularly successful at foraging in colder climates (e.g., Corbet et al., 1993; Heinrich, 1974). To achieve flight, bumblebee thorax muscles must reach approximately 30°C (Heinrich, 1974) accomplished through wing shivering as well as proposed non-shivering mechanisms including the use of mitochondrial substrate glycerol 3-phosphate (Masson et al., 2017) and futile cycling (Staples et al., 2004). With this capacity for thermogenesis, the secondary benefit of nest temperature (T_n) regulation arises. Social bee T_n is therefore maintained within narrow thermal windows of approximately 34-36°C for honeybees (Medrzycki et al., 2010; Stabentheiner et al., 2021, 2010) and 30-33°C for bumblebees (Barrow and Pickard, 1985; Heinrich, 2004; Schultze-Motel, 1991; Vogt, 1986a). Deviations from these optimal nest temperatures lead to detrimental effects on individuals within a colony. For instance, reductions in pupal-stage rearing temperature are well documented to result in impairments to the dance communication, olfactory senses, and short-term memory of adult honeybees (Groh et al., 2004; Jones et al., 2005; Tautz et al., 2003; Wang et al., 2016) and elevated temperatures of 3°C can prevent pupae from emerging as adults (Groh et al., 2004). Larvae also represent a crucial stage of bee development, requiring incubation and feeding by workers to maintain their growth and development to reach the pupal stage (Heinrich, 2004). Previous works document that larvae reared under suboptimal thermal conditions experience higher adult mortality in honeybees (Medrzycki et al., 2010) as well as stunted growth and wrinkled wings in bumblebees (Heinrich, 2004). In a eusocial termite, larvae are also more sensitive to high temperature than adults with a 2 to 3°C lower CTmax, in part attributed to the more constant thermal environmental found in their subterranean nest (Mitchell et al., 1993). The effects of temperature that exceed optimal nest conditions, however, are less explored. Cook et al. (2016) provide evidence that larvae play an influential role in the thermoregulatory behaviours of adult honeybees under elevated temperatures, thus indicating that the larval stage of bee development is thermally sensitive and requires careful nest thermoregulation by the colony. The numerous negative effects on growth and development that result from fluctuations in nest temperature, illustrate that collective thermoregulation is vital for bee colonies to maintain thermal stability within their nests.

Maintaining narrow T_n ranges involves collective thermoregulation. When T_a falls, bees facilitate conductive heat transfer towards their abdomen pressed against brood clumps (see Heinrich, 2004, 1976) resulting in incubation. An individual bumblebee queen incubating her brood at temperatures 10°C or lower have a metabolic rate similar to free flight (see Heinrich 2004). For honeybees, colony metabolic rates triple when temperatures drop from 30°C to 20°C, with bees forming tight clusters to prevent dissipation of metabolic heat as T_a cools (Kronenberg and Heller, 1982). When T_a rises, behavioural wing fanning acts to reduce colony temperatures through evaporative and convective heat loss (Heinrich, 2004; Vogt, 1986a). When T_a rises above optimal values, the incidence of honeybees fanning increases and individuals disperse to facilitate heat loss; metabolic rates simultaneously tend to decrease as temperatures approach 40°C (Kronenberg and Heller, 1982). Small bumblebee colonies (10-42 workers) exposed to temperature lower than 31°C increased the incidence of incubation behaviour and colony metabolic rate tripled below 10°C (Vogt, 1986a). Temperatures above optimal range resulted in higher percentages of workers fanning and a 20% increase in colony metabolic rate (Vogt, 1986a). The energetic burden of maintaining nest temperature in cold conditions is clear in both groups, but the energetic implication of fanning remains unclear as large honeybee colonies (1500-2500 workers) show no clear effect of warm temperatures on colony metabolic rate (Kronenberg and Heller, 1982), and small bumblebee colonies (10-42 individuals in 2 colonies of *B. impatiens* and 1 *Bombus affinus*) show a slight increase (Vogt, 1986a). The size of the colonies and the conditions experienced (presence or absence of insulation) may impact the energetic cost of coping with warm environmental conditions.

This study’s main objective was to characterize the upper temperature tolerance limit of a eusocial endothermic poikilothermic insect, *B. impatiens*, at the individual and colonial level. We first determined the relative thermal tolerance of adult colony castes, also incorporating the larval stage of development into our study to determine if a juvenile stage may present as a thermally sensitive “weak link” when compared to adults. Larvae are predicted to be more sensitive to high temperature as observed in eusocial termites (Mitchell et al., 1993), and studies used this developmental stage to successfully compare CTmax between juvenile and adult insects (e.g., Davison, 1969; Klok and Chown, 2001; Li et al., 2019). Second, the energetic costs of thermoregulation for typical-sized bumblebee colonies of 200 or more workers were measured. The energetic cost of thermoregulation was predicted to rise as T_a deviates away from optimal T_n. The presence of insulation on colony thermoregulation was also investigated as small colonies better maintained T_n in such conditions (Vogt, 1986b). The success of colony thermoregulatory efforts in maintaining T_n was monitored by measuring the nest temperature amongst the brood clumps.

2. Methods

2.1. Bee colonies and holding conditions

The Common Eastern bumblebee, *Bombus impatiens*, is a native underground nesting species with a wide North American distribution (Colla et al., 2014). Commercial colonies of this species were purchased from Biobest Canada Ltd. (Leamington, ON, Canada) to use in both CTmax and whole-colony thermoregulation experiments. All colonies were contained in the supplier's housing boxes in a room maintained at approximately 25°C on a 12h:12h light:dark photoperiod. BIOGLUC® sugar solution from the supplier was available *ad libitum* to colonies and pollen was provided every second day. Individuals used in CTmax experiments were sampled randomly from multiple colonies housed within the lab. An additional ten colonies were used specifically for whole-colony respiration testing (see supplementary material for details).

2.2. Individual CTmax

CTmax was determined using the thermolimit respirometry method (Lighton and Turner, 2004). Mature individual workers, progeny drones and queens, as well as similar-sized late instar larvae that were removed from individual pollen cell (see supplementary material for details), were collected from multiple colonies maintained in the laboratory and weighed on an analytical balance prior to being transferred into a 20 ml glass respirometry chamber. The potential effect of colony was tested for a subset of individuals where no effect was found and not further considered in the analyses (supplementary material). The chamber was placed in an activity detector to monitor bee movements via infrared detection [AD-1; Sable Systems International (SSI), Las Vegas, NV, USA], which in turn, was contained within a temperature-controlled cabinet (PTC-1, SSI). A copper coil was used to allow incurrent air from the flow-through respirometry system to equilibrate with the temperature inside the cabinet prior to entering the respirometry chamber. A FOXBOX Respirometry System (SSI) was used to push air, scrubbed free of water using a drierite column, at a rate of approximately 50 ml min^{-1}. The CO_2 production rate of the animal was measured.

A temperature ramping protocol was used to identify CTmax and each adult bee was subjected to the following temperature sequences using a PELT-5 temperature control unit (SSI): a 20 min soak period at 25°C to allow the bee to equilibrate within the respirometer; a ramping period of 0.25°C min^{-1} until a maximum of 56°C was reached; a subsequent soak period at 56°C for 25 min; a final ramping phase to reduce temperature by 2.0°C min^{-1}. The total ramping protocol elapsed for 190 min. Thermolimit respirometry was also used to determine CTmax of larvae where movement could also be detected using the AD-1 infrared detector. Larvae were not as active as adults and a 10-minute initial equilibrium phase was used. Respirometry, temperature and activity outputs were obtained using an analog to digital converter (UI2, SSI) and acquired using Expedata (SSI). Activity and respiratory CTmax were estimated using the absolute difference sums (ADS) method described by Lighton and Turner (2004). This approach led to difficulties identifying respiratory CTmax of adult castes (see also Vorhees and Bradley, 2012 and supplementary material for details and examples), so we used an alternative approach, the cessation of spiracular activity (CSA), known to yield indistinguishable CTmax values in other insect species (Vorhees and Bradley, 2012). For larvae, only activity CTmax could be determined as respirometry traces did not provide the usual cues (see supplementary material); activity CTmax is commonly used to determine larval thermal tolerance (Cooley et al., 2016; Li et al., 2019; Mitchell et al., 1993). Furthermore, given that respiratory- and activity- CTmax (Kovac et al., 2014; Lighton and Turner, 2004; Vorhees et al., 2013) or CTmin (MacMillan et al., 2012) are highly correlated and not significantly different, we are confident that activity-CTmax alone remains a relevant metric for quantifying larval thermal tolerance.

2.3. Whole-colony thermoregulation

Five colonies were used for experiments where the insulating cotton batten supplied with the colony was removed for visual observation of the colony after each experiment. Removing the insulation disturbed the nest and colonies were allowed two weeks to recover. An additional set of five colonies were used with the insulating cotton batten remaining in place. These colonies were used for the experiment five days after arrival to obtain colonies approximately the same size of those used for the uninsulated group. Colonies sizes were measured after experimentation and averaged 316±17 (SE) workers each.

To record internal nest temperature, a thermochron iButton® (iButtonLink Technology, Whitewater, WI, USA) was placed among the brood cells with developing larvae on the day of experimentation for uninsulated colonies. For insulated colonies, this was done on the day of delivery to minimize the disturbance to the hive. Internal colony temperature was sampled at 2-minute intervals for the duration of the experiment.

The metabolic rate of the colony containing both adults and brood was measured using flow-through respirometry. A single colony within its housing container and accompanying sugar solution was placed in a 30 L plexiglass chamber. Air was pulled from the chamber using a total of three pumps connected in parallel: two SS-3 Gas Analyzer Subsamplers (SSI) with flow rates of approximately 2 L min^{-1} and one FlowBar 8 Multichannel Mass Flow Meter (SSI) with a flow rate of approximately 2.5 L min^{-1}. Outflow was then combined, totalling between 6-6.6L min^{-1}, which was confirmed using a rotameter, and subsampled at 0.2 L min^{-1} to analyze CO_2 concentration (ppm) using Li-7000 CO_2/H_2O analyzer (LI-COR, Inc. Lincoln, NE, USA).

Temperature treatments were obtained by placing the respirometry chamber containing the bee colony within a temperature-controlled incubator (VWR International). Each single colony underwent 6 temperature treatments, one temperature per day, in the following sequence: 25, 5, 35, 15, 30 and 40°C. This sequence was chosen to vary the degree of thermal challenge from day to day, ending with the most challenging treatment at 40°C that induced damaged to the colony. The temperature was held constant for a total of 4 hours during respirometry measurements. The first 60 minutes of each measurement were considered an equalization period for colonies to adjust to experimental conditions and was not included in the analysis. Following the 40°C treatment, the entire colony was sacrificed by placing it in a -80°C freezer, the number of workers were counted and their total mass measured.

Respirometry measurements were collected using Expedata Analysis Software (SSI) and colony metabolic rate was expressed as the rate of CO_2 production per gram (VCO_2 ml hr^{-1}g^{-1}). Colony metabolic rate was expressed as the maximum rate corresponding to the 10 min period with the highest CO_2 production, in addition to the average colony metabolic rate per hour over the last three-hours of measurement. Average T_n was calculated over the last three-hours of measurement as well.

2.4. Data analysis

Statistical analyses were performed using R (R Core Team, 2014) and values are reported as mean ± standard error of the mean. Differences in CTmax measurements among castes were tested using ANCOVAs with body mass as a covariate. The final model presented in the results was simplified by first removing the non-significant interaction term. The simplified model including castes and body mass had non-normally distributed residuals that could not be resolved using data transformations. We confirmed that body mass had no effect on CTmax within each caste through linear regressions and further removed this term from the model. The effect of castes on CTmax was finally tested using a nonparametric Kruskal-Wallis test given that the normality assumption could not be met. The difference in between activity and respiratory CTmax was determined using the Wilcoxon signed-rank exact test.

Whole-colony data analyses were conducted using the lme4 and lmerTest packages (Bates et al., 2015; Kuznetsova et al., 2017) to perform linear mixed models. To accommodate the assumptions of normality and homogeneity of variance, maximum and mean hourly colony metabolic rate were log-transformed, while nest temperature was raised to the third power. Linear mixed models were conducted to test for differences in colony maximum metabolic rate and nest temperature using the REML estimation method where colonies were considered as a random variable and ambient temperature and insulation factor were fixed effects. Mean hourly metabolic rate was also tested for changes over time where hour (1st, 2nd and 3rd) was further added as fixed effect in the model. Tukey pairwise comparisons were performed for each analysis.

3. Results

3.1. Bumblebee caste CTmax

Activity-CTmax differed between adults and larvae (Kruskal-Wallis χ^2=31.767, df=3, *P*<0.001; Fig. 1A) with higher activity-CTmax for adult castes (drone: 45.65 ± 0.37˚C (n=21), queen: 46.39 ± 0.32˚C (n=19), worker: 46.19 ± 0.14˚C (n=21) and approximately 2.5˚C lower for larvae (43.44 ± 0.32˚C (n=20)). Adult casts did not differ in activity-CTmax values (*P*≥0.648), and they were all significantly higher than larvae (*P*≤0.001).

Respiratory-CTmax that could be determined for workers, drones and queens was found to be significantly influenced by caste (Kruskal-Wallis χ^2=9.4371, df=2, *P*<0.001; Fig. 1B) where drones (47.18 ± 0.27˚C) had significantly higher respiratory-CTmax compared to workers (46.53 ± 0.13˚C; *P*=0.008) but not to queens (46.57 ± 0.27˚C; *P*=0.128). Respiratory-CTmax was greater than activity-CTmax in drones (Z=3.98, *P*<0.001) and workers (Z=3.98, *P*<0.001), but not for queens (Z=0.282, *P*=0.78).

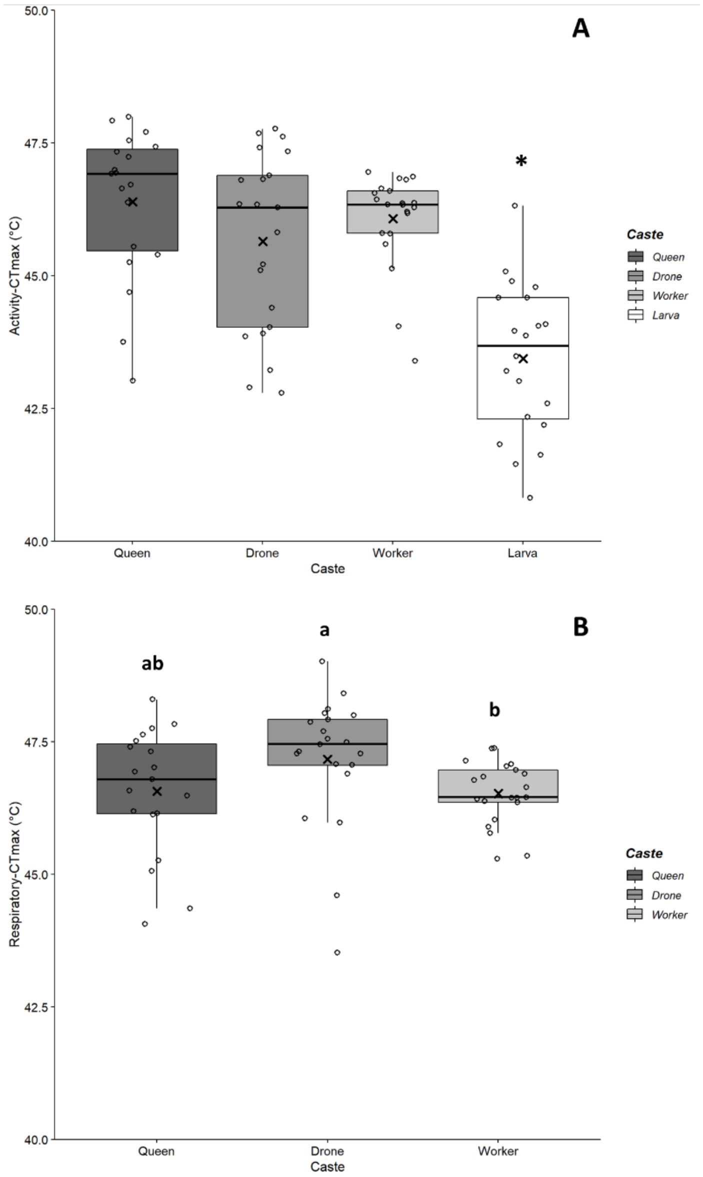

Fig. 1. CTmax comparisons across bumblebee (*B. impatiens*) castes. Individual queens, drones, workers and larvae were ramped at 0.25°C min^{-1} to determine their critical thermal maxima (CTmax). A) The temperature at which muscular control is lost (activity-CTmax) is significantly lower for larvae when compared to all adult castes (**P*≤0.001). B) The temperature at which spiracular control is lost (respiratory-CTmax) differ among adult castes with drones having higher respiratory-CTmax than workers as indicated by different letters (*P*=0.008). In both panels, box plots represent the 25th and 75th percentiles, the error bars the range of values, the black bar across indicating the median value and the x representing the mean.

3.2. Whole-colony thermoregulation

The mean number of individuals in colonies measured at the end of the experiment differed between the uninsulated colonies averaging 284±15 workers and the insulated colonies with an average of 348±22 workers ($t(7.17)=2.44$, $P=0.044$). This difference in size was due to one colony that was substantially larger than the other colonies with 404 individuals; analysis conducted without this large colony show no difference between groups ($t(5.71)=1.93$, $P=0.105$). All further analyses were done both with and without this large colony to assess its influence on the results.

Maximum colony metabolic rate was affected by T_a and the presence of insulation in the colony (Fig. 2; T_a: $F_{5,45}=16.88$, $P<0.001$; insulation: $F_{1,8}=6.24$, $P=0.037$); the interactive effect between T_a and insulation was not significant and was removed from the model. The maximum metabolic rate of insulated colonies was approximately 15% lower than uninsulated colonies ($P=0.037$). When comparing the maximum metabolic rate of colonies at the various T_a (Fig. 2), the lowest values were observed at 25°C and 30°C and did not differ from one another ($P=0.927$). As T_a decreases from 25°C down to 5°C and 15°C, the maximum metabolic rate increased by approximately 26% and 31%, respectively ($P\leq0.001$). Similarly, when T_a rose from 30°C up to 35°C and 40°C, the maximum metabolic rate also increased by around 22% and 36%, respectively ($P\leq0.001$). The absence of the large, insulated colony did not alter the overall statistical outcome of maximum colony metabolic rate (T_a: $F_{5,40}=13.815$, $P<0.001$; insulation factor: $F_{1,7}=6.39$, $P=0.039$).

The average hourly metabolic rate of the colonies showed a significant interaction between the temperature treatment and the hour measured, and the presence of insulation was not significant and removed from the model (Fig. 3; T_a: $F_{5,9}=31.47$, $P<0.001$; hour: $F_{2,108}=0.51$,

$P=0.603$; $T_a \times$hour: $F_{10,108}=40.37$, $P<0.001$). At T_a of 5-30°C, there were small differences in the metabolic rate over the three consecutive hours of measurement (15°C, 1st to 3rd hour: $P=0.044$; 30°C, 1st to 3rd hour: $P=0.002$). Larger changes were observed for colonies exposed to 35°C, where it increased by 17% from the 1st to the 2nd hour ($P<0.001$), 19% from the 2nd to 3rd hour ($P<0.001$). Colonies exposed to 40°C showed a continuous decline in metabolic rate falling by 17% from the 1st to 2nd hours ($P<0.001$) and 12% from the 2nd to 3rd hours ($P<0.001$). Removing the large, insulated colony from analysis did not change the overall results for hourly metabolic rate (T_a: $F_{5,136}=52.125$, $P<0.001$; hour: $F_{2,136}=0.1393$, $P=0.870$; T_a-hour: $F_{10,136}=6.593$, $P<0.001$).

Ambient temperature, T_a, and insulation had a significant effect on nest temperature, T_n, (Fig. 4; T_a: $F_{5,53}=106.852$, $P<0.001$; insulation: $F_{1,53}=4.409$, $P=0.041$); but the interactive effect between T_a and insulation was not significant and removed from the model. Despite a significant overall effect, pairwise analysis revealed that mean T_n of insulated colonies was not different from uninsulated colonies ($P=0.069$). Excluding the large, insulated colony from the analysis slightly alter the statistical outcomes of colony T_n where the overall effect of insulation on T_n became insignificant (T_a: $F_{5,47}=91.882$, $P<0.001$; insulation: $F_{1,47}=2.431$, $P=0.126$). Examining T_n between various T_a conditions, it did not differ between 15, 25 or 30°C with T_n of 32.3, 32.0 and 33.0°C, respectively ($P>0.05$); these temperature values falling within the range of optimal T_n (Fig. 4). When T_a was reduced to 5°C, T_n declined by approximately 3.0°C when compared to T_n at 30°C ($P\leq 0.001$). At T_a of 35°C and 40°C, T_n increased by 2.0°C and 6.9°C, respectively ($P\leq 0.003$). At 35°C, both insulated and uninsulated colonies experienced T_n that was dependent on the hour of measurement (uninsulated, hour: $F_{2,8}=53.51$, $P<0.001$; insulated, hour: $F_{2,8}=151.40$, $P<0.001$) where T_n increased over each hour of exposure ($P<0.001$) for a total increase from the first to the third hour of 1.5 and 1.9°C, respectively. Similarly, at 40°C, T_n was also dependent on hour (uninsulated, hour: $F_{2,8}=317.79$, $P<0.001$; insulated, hour: $F_{2,8}=73.53$, $P<0.001$) where T_n increased by 1.5 and 1.8°C, respectively.

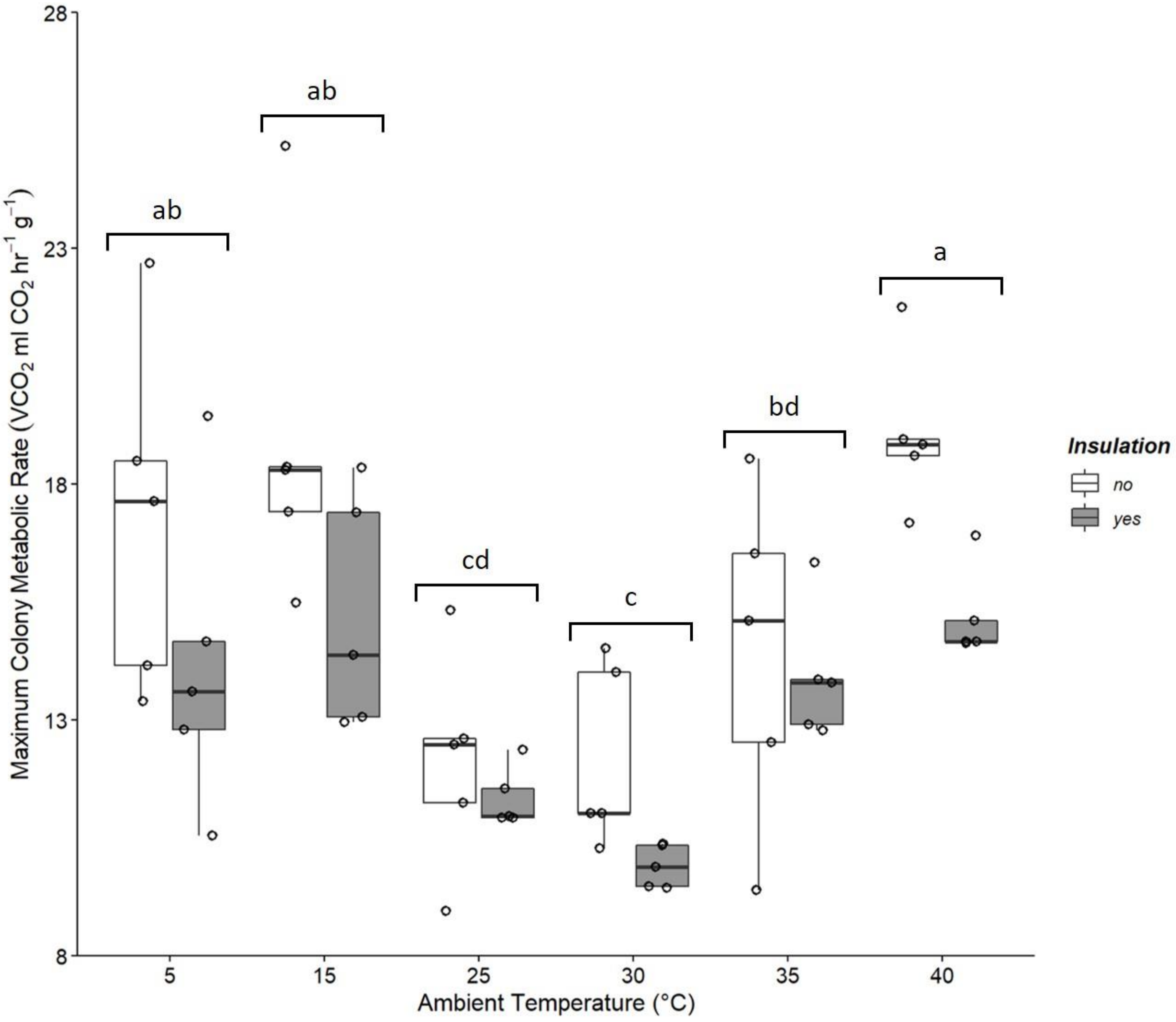

Fig. 2. Ambient temperature affects the colony metabolic rate of insulated and uninsulated *B. impatiens* colonies. Flow-through respirometry was used to assess the maximum metabolic rate of insulated and uninsulated bumblebee colonies during a three-hour exposure to various ambient temperatures (T_a). Colony metabolic rate increased significantly above and below Ta of 25 and 30ºC ($P\leq0.005$). Metabolic rates differed between T_a groups (n=10) which do not share letters (a-d). Colonies with insulation have lower colony metabolic rate ($P=0.037$). Boxplots each represents percentiles, with the black bar across indicating the median value. Also present are the individual data points for each colony tested.

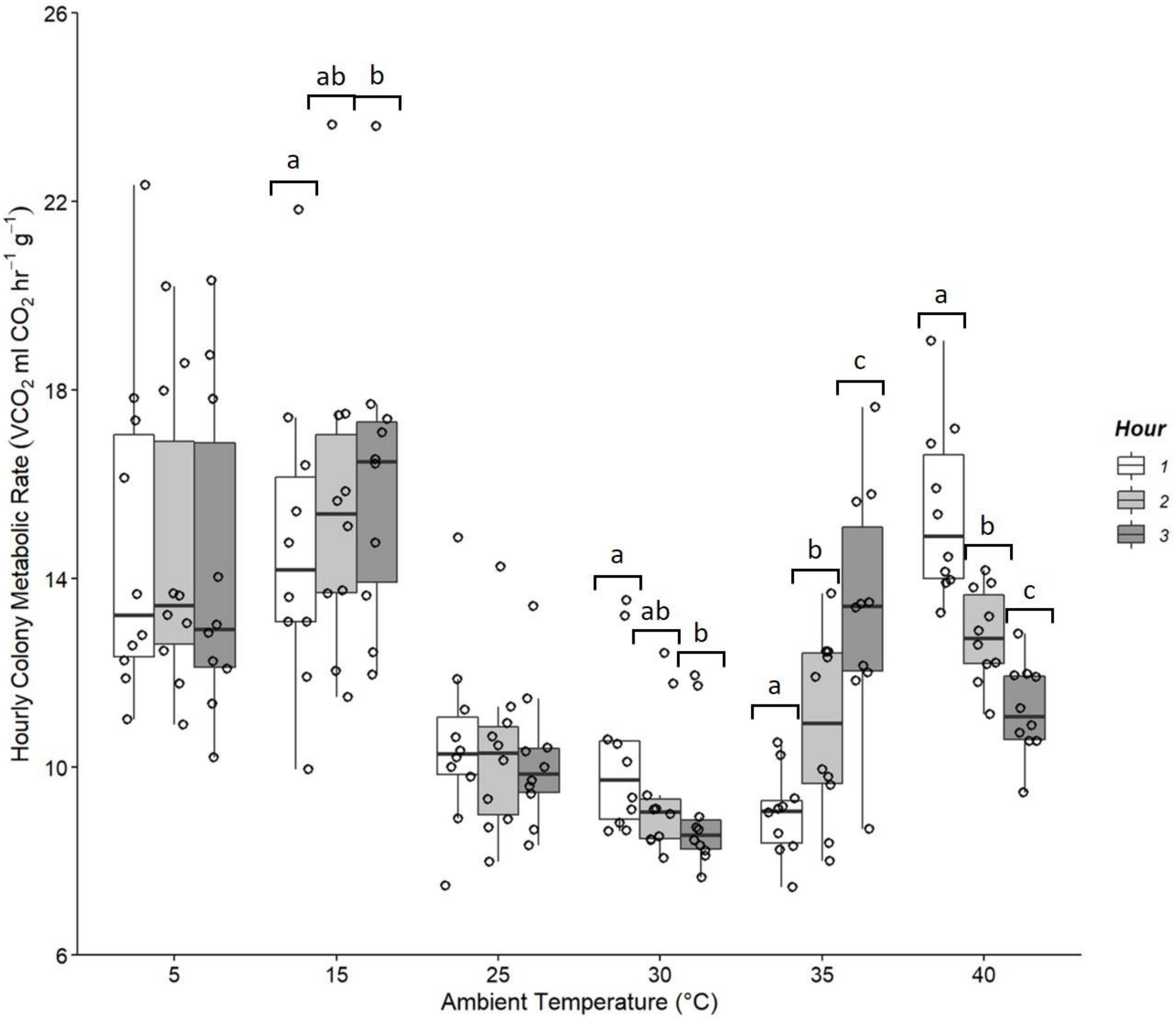

Fig. 3.3. Colony metabolic rate changes over the duration of exposure at high ambient temperature in *B. impatiens*. Flow-through respirometry was used to assess the metabolic rate of whole bumblebee colonies during a three-hour exposure to various ambient temperatures (T_a). At 30°C and below, colony metabolic rate varies little if at all over the three-hour exposure period (15°C, *P*=0.044; 30°C, *P*=0.002). At a high T_a of 35°C, colony metabolic rate increases during each hour of exposure (*P*<0.001), whereas at 40°C, colony metabolic rate is initially elevated but decreases after each hour of exposure (*P*<0.001). Significant differences between hours are presented with the letters a to c within each temperature group. Boxplots represent percentiles with the black bar across signalling the median value. Also present are the individual data points for each colony tested. A sample size of n=10 colonies was used for each group.

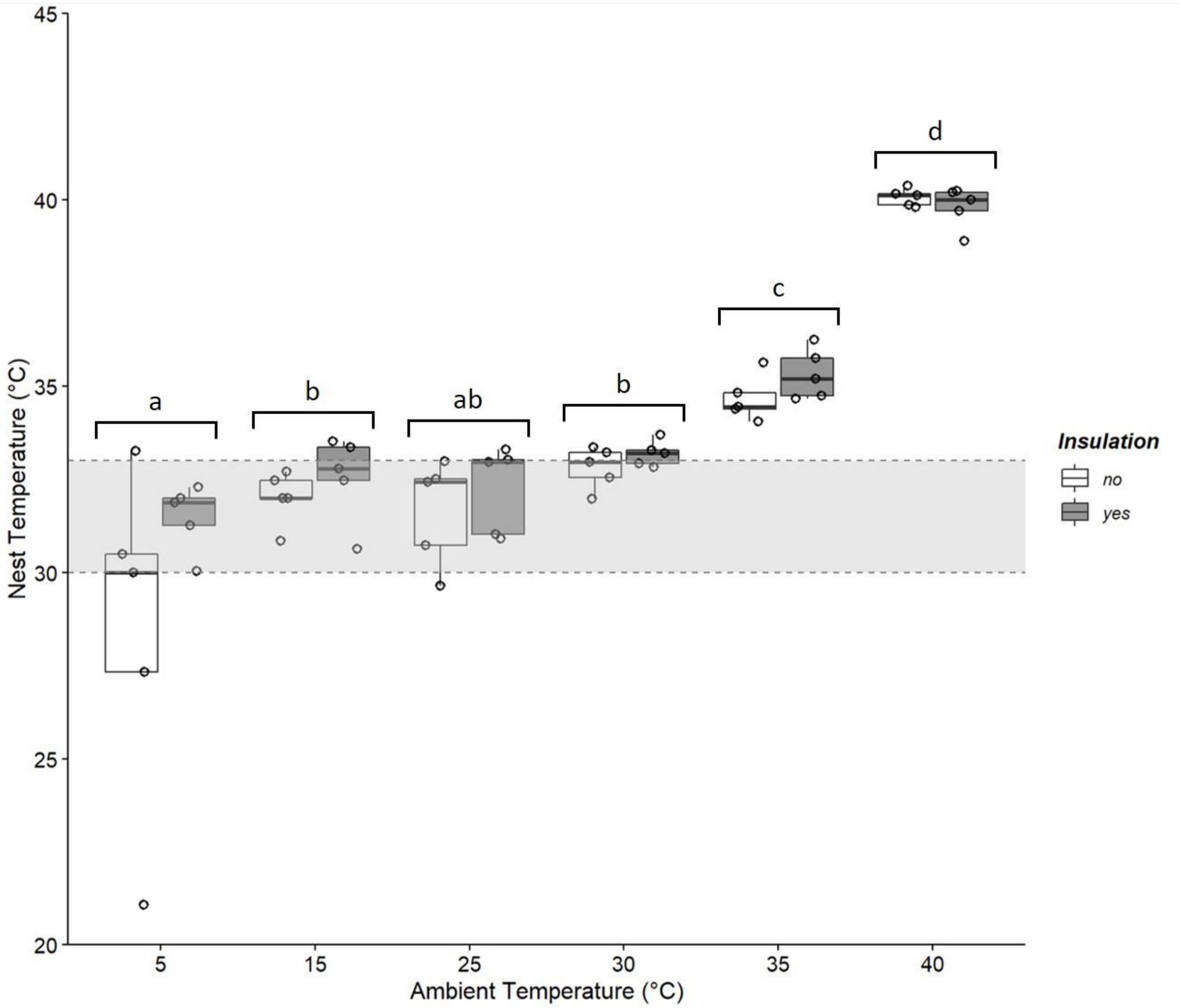

Fig. 3.4. Nest temperature of *B. impatiens* colonies, with and without insulation, exposed to various ambient temperatures. Nest temperature (T_n) represents the average temperature maintained by *B. impatiens* colonies calculated across a three-hour exposure period to various ambient temperatures (T_a) for uninsulated (n=5) and insulated (n=5) colonies. An optimal T_n of 30-33°C (see section 1), represented by the horizontal shaded area on the graph, is achieved for colonies exposed to 30°C and below. At T_a which exceed this idea range (35°C and 40°C), T_n rose to ambient, being significantly higher than T_n achieved at 30°C in both cases ($P \leq 0.003$). Insulated colony T_n was not significantly different than uninsulated T_n ($P=0.069$). Boxplots each represents percentiles, with the black bar across indicating the median value. Also present are the individual data points for each colony tested. Nest temperature means which differ between T_a groups who do not share letters (a-d).

4. Discussion

Current efforts attempt to relate physiological thermal limits of species with their thermal environment and ultimately geographical distribution. However, for social endothermic poikilotherms, such as bumblebees, understanding their response to thermal variation within the environment involves investigation into both individual- and colony-level thermal tolerance. The present study first assessed the acute upper thermal tolerance limits of *B. impatiens* castes and developmental stages in order to identify which members of a colony were the most thermally sensitive. We show that adult castes have similar CTmax and the larvae are the most sensitive, highlighting the importance of T_n regulation. The energetic costs of nest thermoregulation for peak-season sized colonies increase as T_a decreased and increased away from optimal T_n range, the presence of insulation reducing those costs. At high T_a, colony metabolic rate changed substantially over time indicating challenges in thermoregulating. Colonies experiencing high T_a failed to regulate T_n that increased to T_a. Together, we here show the central challenges of regulating nest temperature at high environmental temperature and protecting the thermally sensitive larvae.

4.1. Bumblebee caste CTmax

The activity-critical thermal maximum of *B. impatiens*, more specifically the temperature at which muscular control was lost, was found to be on average 46.03 ± 0.18°C for adults. The respiratory-CTmax showed very similar values, although slightly higher for workers and a more pronounced difference observed for drones. For the larval developmental stage, only activity-CTmax could be determined and larvae are more susceptible to high temperature than adults with CTmax values approximately 2.5°C lower. These findings for adults are consistent with a previous study that determined *B. impatiens* workers to have a CTmax of 46.07°C using the righting response technique (Hamblin et al., 2017). Conversely, other works found differing values for this species, ranging from 50 to 53°C for workers and queens when using the onset of spasm methodology (Maebe et al., 2021; Oyen and Dillon, 2018). The methodology used to determine thermal limits, including the method of assessing end-point temperature (e.g., thermolimit respirometry, onset of spasms or righting response) and ramping rate, is well known to affect the values obtained (see Gonzalez et al., 2020; Lighton and Turner, 2004; Terblanche et al., 2007), yet the merit of CTmax testing lies within the relative differences observed between individuals or groups tested. For example, the lack of difference in CTmax observed between workers and queens has also been reported on other species of bumblebees (Maebe et al., 2021), while neither age of the adult, mass, feeding or acclimation temperature significantly alter the temperatures which bees are capable of tolerating (Gonzalez et al., 2020; Oyen and Dillon, 2018). Having multiple metrics of CTmax also appears useful to detect the central emerging patterns as different measures sometimes yield slightly different outcomes such as drones that differ from workers for respiratory-CTmax but not when activity-CTmax is used. Furthermore, interspecific comparisons show that bees which nest in cavities have lower thermal tolerances versus species that nest in stems or in the ground (Hamblin et al., 2017), indicating that the thermal conditions experienced help govern insect thermal tolerance, just as low altitude species and populations of bees have been found to possess higher upper thermal limits than those found at higher elevations (Gonzalez et al., 2020; Oyen et al., 2016). Not only that, but CTmax also demonstrates predictive power for determining insect responses to warming both experimentally and within communities (Diamond et al., 2012; Hamblin et al., 2017) providing support for its relevance in understanding species' responses to environmental thermal challenges.

Baudier and O'Donnell (2017) indicate that most studies do not account for the negative effects temperature poses on the most thermally sensitive members of insect colonies and call for an inclusion of minimal thermal tolerance when considering a species' vulnerability to thermal stress. This "weak link" hypothesis is discussed in the context of worker castes only, but it is also important to consider additional castes or developmental stages as potential weak links within a species. Previous works review how juvenile, young adults and senescent insects differ in their thermal tolerance ranges (Bowler and Terblanche, 2008), emphasizing the importance of also including juveniles within studies on social insect thermal tolerance. Larvae have previously been used to compare the thermal tolerance of a juvenile stage to that of adults. For example, kelp fly larvae are more heat tolerant likely due to limited behavioural response to temperature resulting from restricted mobility (Klok and Chown, 2001). Similarly, wood wasp larvae are more tolerant than adults due, in part, to their emergence time during the year (Li et al., 2019). In contrast, the beetle larvae studied in Vorhees and Bradley (2012), demonstrate similar heat tolerances as pupae, yet both life stages are less tolerant than adults, potentially in response to the microclimate conditions experienced during each life stage. The reduced thermal tolerance of larvae observed in subterranean species such as termites may also be reflecting the nest microclimate conditions (Mitchell et al., 1993). Thus, in social hymenopterans, where colonies care for offspring and create an ideal thermal microclimate within the nest, larvae represent a vulnerable stage in the life cycle of species like the bumblebees tested within our study, and additional developmental stages should be included for a comprehensive assessment of the "weak links" of the species. As such, colony thermoregulation is vital in the protection of thermally sensitive larvae, but its associated costs and success are dependent on the T_a encountered.

4.2. Whole-colony thermoregulation

4.2.1. Maximum whole-colony energy expenditure

The energy expenditure of colonies of *B. impatiens* of sizes corresponding to peak season increases as T_a deviates away from the range of optimal T_n, at both the lower and upper thermal extremes. Colonies that experience 25°C and 30°C are within or near the range of optimal T_n and have the lowest colony metabolic rate as they likely expend less thermoregulatory effort to maintain nest conditions. This is consistent with the findings of Vogt (1986a) using small colonies of two bumblebee species.

The change in energy expenditure of bee colonies is likely attributed to increased incidence of behaviours associated with communal thermoregulation, especially when exposed to low temperatures. Honeybees cluster to incubate as T_a drops, elevating colony energy expenditure (Kronenberg and Heller, 1982). In bumblebees, a 50-80% incidence of incubation was reported when T_a dropped below 20°C, accompanied by an approximately 3-fold increase in energy consumption as T_a reached 3°C (Vogt, 1986a). In comparison, our larger colonies experienced an increase in metabolic rate of 1.3-1.5 times when T_a fell to 5-15°C. Thus, colony size probably has a strong influence on the cost of thermoregulation and remains to be studied systematically. Our study using colonies of over 200 workers shows a reduced cost compared with Vogt (1986a), indicating the likely importance of thermal conductance with more individuals thermoregulating and possibly clustering at low temperatures (Rivière, 2012). Furthermore, insulated colonies had overall reduced metabolic rates in comparison to colonies lacking insulation. In colder conditions, insulation likely reduces heat loss and colony energy expenditure associated with thermogenesis.

High T_a also poses an energetic challenge and colonies expend more energy in such conditions, which may in part be due to mechanisms mobilized to dissipate excess heat. Our large colonies demonstrated a 22-36% increase in energy expenditure when T_a was greater than 30°C. Vogt (1986a) also described a 20% elevation in metabolic rate when T_a rose from 31 to 39°C. At T_a of 35 and 40°C, T_n increases and reaches T_a, which can impact the resting metabolic rate of individuals that cannot regulate their body temperature, such as developing brood. The elevation in resting metabolic rate with temperature documented in the honeybee shows that it is not straightforward and linear (Kovac et al., 2007), but using a simple linear Q10 effect of 2 to 3 to approximate the impact of increased temperature predicts that larvae going from 33 to 35°C would lead to a 20 to 30% increase in metabolic rate. This proportional increase coincides with the elevation in colony metabolic rate observed, but the presence of a large number (>200) of endothermic workers in our colonies must, to some extent, contribute to the increased colony metabolic rate. Small bumblebee colonies will allocate between 20 and 100% of their adult workforce towards the fanning behaviour (Vogt, 1986a) in an attempt to thermoregulate the nest. The fanning behaviour involves flight muscle contraction that can generate heat as a by-product and contribute to temperatures experienced within the colony. Stabentheiner et al. (2021) showed the complex and dynamic changes observed in colonial thermoregulation in large honeybee colonies, where at high T_a (40°C) many workers leave the nest to reduce overall heat production. The contribution of fanning to the colony energy expenditure should be assessed further to evaluate the temperature range at which it is most effective where heat dissipation is greater than the heat gain associated with muscle contraction.

Colonies exposed to high T_a exhibit changes to their energy expenditure over time, pointing to the importance of fanning behaviour engaged by adults. When exposed to 35°C, a temperature slightly above optimal T_n, colonies increase their metabolic rate over a three-hour period (Fig. 3). In contrast, the highest tested temperature of 40°C, imposes a cost that does not appear sustainable because colony metabolic rate decreases over time. Our results coincide with observations in the literature where fanning incidence in honeybee and bumblebee colonies increases at temperatures that exceed 30°C (Kronenberg and Heller, 1982; Vogt, 1986a). The recruitment of fanners occurs when individual worker thermal thresholds are surpassed for the behaviour to initiate. When heated to 30°C, *B. terrestris* exhibit thresholds between 27 and 28.7°C (Weidenmüller, 2004), yet individuals may each have differing thermal triggers, resulting in either an immediate or delayed response (Jandt and Dornhaus, 2014). The increase in metabolic rate over time observed at 35°C for our colonies may be indicative of more individual thresholds being triggered as the thermal threat persists. Accordingly, workers were observed vigorously fanning at the end of 35°C trials. Nonetheless, this is confounded by the concurrent increase in nest temperature also observed over the three-hour measurement period that may affect the brood metabolic rate. The contrasting pattern observed at 40°C, where T_n also rose gradually over the three-hour period, helps evaluate the contribution of fanning to the whole-colony energy expenditure. Despite the gradual increase in T_n , the whole-colony metabolic rate decreases gradually. A large number of individuals likely reached their thermal thresholds early, coinciding with initial high metabolic rates within the first hour. As T_a of 40°C persisted, the hive structures sustained heat damage with workers abandoning their fanning behaviour and attempting to leave the nest box, thus decreasing the colony metabolic rate over time. This reduction in colony metabolic rate over time, despite an increase in T_n over the same period, supports the contribution of the fanning behaviour to the colony energy expenditure. The T_n

encountered which matched the T_a of 40°C, appears beyond the capacity of collective thermoregulation and is approaching the thermal limits of larvae found via activity-CTmax.

Bumblebee workers in a colony collectively act as a superorganism to thermoregulate T_n for the larvae, incurring energetic costs. When subjected to various T_a conditions, colony metabolic rates create a pattern that is reminiscent of vertebrate endothermic homeotherms. The optimal T_n range of colonies resembles the thermoneutral zone where the metabolic costs to regulate body temperature is minimal. For the present study, this range corresponds to 25-30°C where the lowest metabolic rates were observed. Below the thermoneutral zone, endothermic homeothermic animals must increase metabolic rates to maintain body temperature. The increase in energy expenditure above the 25-30°C thermoneutral zone can be due to the combined effects of increased nest temperature on ectothermic individuals within the colony, mostly the brood, but also mechanisms mobilized to thermoregulate, such as fanning. The changes in colony metabolic rate observed over the three-hour measurement period suggest that fanning contributes to the observed increase in energy expenditure at 35 and 40°C. The increase in colony metabolic rate over consecutive hours at 35°C is more substantial than the increase in nest temperature observed over the same period, suggesting greater investment in fanning efforts. Furthermore, at 40°C there is also an increase in nest temperature over the three consecutive hours of measurements, but colony metabolic rate shows a decrease over the same time period, indicating that colony metabolic rate is decoupled from nest temperature. We therefore suggest that a large part of the increased energy expenditure at 35 and 40°C is associated with the fanning behaviour. These comparisons highlight the importance of social cooperation in bee colonies, especially in consideration of the apparently vulnerable larvae within the nest.

4.2.3. Internal colony nest temperature

Overall, bumblebee colonies were successful at maintaining T_n when acute thermal challenges did not exceed that of optimal T_n, 30-33°C (*B. impatiens* 28-32°C, Vogt 1986a; *Bombus lapidarius* 31.7±1.0°C, Schultze-Motel 1991; *B. terrestris* 32.3±0.4°C, Weidenmuller et al. 2002). At 25 and 30°C, optimal T_n was achieved with the least energetic effort, while at 5°C and 15°C, this energetic cost rose due to incubation efforts as workers attempted to buffer against a drop in T_n. The capacity of *B. impatiens* colonies to maintain optimal T_n at low T_a further exemplifies how robust bumblebees are against low T_a. For example, the arctic species (*Bombus polaris*) possess exceptional thermoregulatory capacities that enable colonies to maintain T_n at 35°C when T_a falls to 7.5-11°C in the summers (Richards, 1973).

The presence of insulation also had an overall effect on T_n where insulated colonies were about half a degree warmer than uninsulated colonies, although this effect appears to be largely influenced by one larger colony in our study. Nevertheless, other work shows a reduced T_n value in the absence of insulation and also a reduced number of workers and drones by the end of their lifecycles (Vogt, 1986b). Moreover, insulation clearly lowers the overall energetic costs of thermoregulation, reducing the incidence of brood incubation necessary for maintaining T_n (Vogt, 1986b). Our study simulates surface or aboveground nests with and without insulation. Underground nesting sites appear to be the most common across subgenera of bumblebees, including being the preferred nesting strategy of *B. impatiens* (Colla et al., 2014), though surface-level and aboveground nests are also frequented in both wild and artificial or human-made nest sites (Liczner and Colla, 2019). Simulating aboveground nesting sites also holds relevance given that bumblebees, like the *B. impatiens* colonies used in our study, are commercially available for use in greenhouse and garden pollination (Velthuis and Van Doorn, 2006). Nests located aboveground experience wider fluctuations in T_a as demonstrated in a study on *B. impatiens* using empty, artificial nests (Mullan, 2022), and choosing a thermally optimal nesting site implies success in the rearing of bee offspring (Potts and Willmer, 1997; Vickruck and Richards, 2012; Wuellner, 1999). Therefore, understanding how colonies may buffer temperature fluctuations, which can vary widely according to colony size and species (Gradišek et al., 2023), provides insight into whole-colony responses to thermal stress in common nesting locations. On the other hand, high T_a impedes a bumblebee colony's ability to maintain optimal T_n. At 35 and 40°C, T_n was equivalent to T_a, demonstrating that the high energetic costs associated with these temperatures do not result in successful thermoregulation. Similarly, Vogt determined that despite over 50% of the available workforce fanning within small colonies, optimal T_n could not be maintained and was consistently 1 to 2°C higher than T_a between 33 and 39°C. As such, fanning efforts cannot successfully dissipate sufficient heat through evaporative or convective means to lower T_n, ultimately posing potential consequences to the individuals within a colony.

5. Conclusions

The present study, as well as previous works investigating the physiological and behavioural consequences of changes in T_a and T_n, underscore the importance of understanding how both individuals and colonies respond to varying thermal conditions. Responses to temperature differ between individuals and the colony superorganism. The lower thermal tolerance found for larvae, emphasize the need for colonies to thermoregulate against changes within the thermal environment, however, thermal challenges where T_n rises above optimal, may prove to be energetically costly and unsustainable for colonies. Sustainability comes into question when considering that thermal stress decreases brood maintenance (Vogt, 1986a) and negatively impacts foraging activity (e.g., Hemberger et al., 2023; Kwon and Saeed, 2003), leaving fewer individuals available for these essential tasks. Given that both nutritional and thermal stress leads to reduced colony growth (Vanderplanck et al., 2019), elevated energetic costs under high T_a in addition to the inability to successfully thermoregulate at high temperatures, may result in additional strain on colonies reducing growth or causing colony failure if thermally challenging conditions persist over longer periods of time.

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