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RESEARCH ARTICLE

Evaluating the role of social context and environmental factors in mediating overwintering physiology in honey bees (*Apis mellifera*)

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ABSTRACT

In temperate climates, honey bees show strong phenotypic plasticity associated with seasonal changes. In summer, worker bees typically only survive for about a month and can be further classified as young nurse bees (which feed the developing brood) and older forager bees. In winter, brood production and foraging halt and the worker bees live for several months. These differences in task and longevity are reflected in their physiology, with summer nurses and long-lived winter bees typically having large fat bodies, high expression levels of *vitellogenin* (a longevity-, nutrition- and immune-related gene), and large provisioning glands in their head. The environmental factors (both within the colony and within the surrounding environment) that trigger this transition to long-lived winter bees are poorly understood. One theory is that winter bees are an extended nurse bee state, brought on by a reduction in nursing duties in autumn (i.e. lower brood area). We examined that theory here by assessing nurse bee physiology in both the summer and autumn, in colonies with varying levels of brood. We found that season is a better predictor of nurse bee physiology than brood area. This suggests that seasonal factors beyond brood area, such as pollen availability and colony demography, may be necessary for inducing the winter bee phenotype. This finding furthers our understanding of winter bee biology, which could have important implications for colony management for winter, a critical period for colony survival.

KEY WORDS: Autumn, Brood, Fat body, Hypopharyngeal gland, Season, Vitellogenin

INTRODUCTION

In temperate regions, many animal species bypass the winter by entering a distinct physiological state (Cherednikov, 1967; Mohr et al., 2020; Denlinger, 2022). Insect species will enter diapause, which is a state characterized by reduced activity or dormancy, arrested development or reproduction, reduced metabolic activity, increased internal fat reserves, etc. (Denlinger, 2022). Environmental factors such as changes in photoperiod or temperature are typically used as cues for insects to develop a 'winter' phenotype (Beck, 1983; Hodek and Hodková, 1988; Nelson et al., 2010; Tougeron, 2019). Honey bees (*Apis mellifera*) are unique in that they overwinter in a thermoregulating social group (Southwick, 1983). Though they remain active, honey bee workers exhibit a distinct phenotype in the winter, which includes changes in internal energy stores, hormones and gland activity (Döke et al., 2015). Colony demographic structure is also distinct in the winter; the colony becomes broodless as it ceases to rear new bees (Döke et al., 2015). The presence of brood and the care of brood both influence the physiology of individual bees (Amdam et al., 2009b; Smedal et al., 2009). It has been hypothesized that social context and/or environmental conditions influence the production of winter honey bees (Döke et al., 2015), but uncoupling these two factors is challenging.

Honey bees in temperate climates exhibit distinct phenotypes: that of a short-lived (~4 weeks) summer bee or a long-lived (>8 months) winter bee (i.e. diutinus bee) (Winston, 1987). During the warm summer months, workers spend their first ~3 weeks of life performing in-hive tasks such as feeding developing brood (larvae). As they age, these workers make the transition to out-of-hive activities, including foraging for pollen and nectar (Robinson, 1992). Thus, summer bees can be further divided into the phenotypes of 'nurse' and 'forager' bees, respectively (Seeley, 1982). During autumn and the early winter months, temperatures begin to preclude flying outside the hive (Heinrich, 1996), floral resources for pollen and nectar (honey bee food) are greatly reduced, and brood rearing is diminished and/or halts altogether. During this seasonal transition, winter bees are produced. This cohort of generalist winter bees will survive the entire winter, until favorable conditions return the following spring (Seeley and Visscher, 1985; Winston, 1987). During the winter, workers form a cluster and thermoregulate, feeding on stored food resources (primarily nectar stored in the form of honey) for energy (Southwick, 1983).

Summer nurses, summer foragers and winter bees are each physiologically distinct. Summer nurse bees have larger hypopharyngeal glands (which produce secretions that are fed to brood), larger fat bodies, higher vitellogenin (Vg) titers and lower juvenile hormone (JH) titers than summer foragers (Fluri et al., 1977; 1982; Amdam and Omholt, 2003; Steinmann et al., 2015). These differences correspond to nursing tasks; when nurse bees are exposed to brood pheromone, they are primed to consume pollen, thereby growing the provisioning glands in their heads (Corby-Harris et al., 2022), decreasing JH titers and increasing their Vg titers (Le Conte et al., 2001). The nurses provision larvae with royal jelly, a protein-rich secretion from the hypopharyngeal glands in their heads, synthesized from Vg (Snodgrass, 1956; Amdam et al., 2003). Vg, a protein associated with several functions in honey bees (Amdam et al., 2012), is synthesized in the fat body (Exceles, 1974; Chapman, 1998; Amdam et al., 2012) at high rates for the first 10 days of the bee's life (Amdam et al., 2009a; Alaux et al., 2018). The fat body and hypopharyngeal glands both grow during this period with the consumption of pollen (Haydak, 1970; Alaux et al., 2010; Corby-Harris et al., 2022). As nurses continue to feed brood, their Vg levels are diminished as Vg utilization for feeding exceeds production (Smedal et al., 2009), their fat bodies similarly diminish in size (Toth and Robinson, 2005), and their provisioning glands shrink (Hrassnigg and Crailsheim, 1998). Similar to summer nurse bees, winter bees have large, active fat bodies and high Vg titer

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(Fluri et al., 1982), which are a source of energy storage during the long winter months (Arrese and Soulages, 2010), and contribute to antioxidant protection, immunocompetence and longevity (Seehuus et al., 2006; Amdam et al., 2012), respectively. Transcriptional assessments show that fat body gene expression is indeed very similar between summer nurse bees and winter bees (Bresnahan et al., 2022).

It has been hypothesized that the production of winter bees is related to changes in the demographic structure of the colony, resulting in the alleviation of nursing duties or the 'nurses' load' (Maurizio and Hodges, 1950; Eishchen et al., 1982; Omholt, 1988; Amdam et al., 2009b; Smedal et al., 2009). This theory suggests that when brood production slows in autumn, nurses retain their fat stores and high Vg levels and thus winter bees are simply an extreme extension the nurse bee state. Observational and manipulative experiments show a negative correlation between brood population size or brood pheromone and physiological markers of nurse/winter bees (e.g. Vg, fat body size, longevity) (Amdam et al., 2009b; Smedal et al., 2009). However, other work shows that a lack of brood pheromone accelerates the worker bees' transition to foraging (Le Conte et al., 2001). Furthermore, not all studies observed an effect of brood pheromone on the winter bee transition (Eischen et al., 1984).

In addition to reduced nursing load, the summer–winter transition occurs in the context of many other seasonal shifts in environmental conditions. This dynamic system was summarized well in Döke et al. (2015), who suggested that the summer–winter transition is orchestrated by several synergistic factors. For example, reduced temperature and photoperiod have each been associated with a winter-like state (Cherednikov, 1967; Fluri and Bogdanov, 1987a; Huang and Robinson, 1995). Huang and Robinson (1995) showed that reduced temperature and/or photoperiod can reduce JH production (which increases Vg synthesis; Pinto et al., 2000), and Cherednikov (1967) and Fluri and Bogdanov (1987a) both demonstrated that artificial shortening of photoperiod is associated with more winter-like workers with larger fat bodies. Shorter days and colder temperatures also reduce foraging, thereby increasing the number of foragers in the hive. Exposure to forager pheromone represses nurses' transition to foraging (Huang and Robinson, 1992; Leoncini et al., 2004). Middle-aged nurses are also 'pushed' from nursing by younger nurses (high nurse-to-brood ratio) and 'pulled' to foraging by exposure to brood pheromone (Johnson, 2010), so an increase in forager pheromone, reduced brood and fewer emerging nurses may all help winter bees retain a nurse/winter-like physiology. Reduced foraging, together with fewer floral resources in late autumn/winter, also results in less incoming food, which has been linked to winter bee physiology (Mattila and Otis, 2007). Mattila and Otis (2007) showed that by restricting incoming food, brood production (which requires pollen) is reduced, and newly emerged workers of broodless colonies become winter bees (Maurizio and Hodges, 1950; Fluri et al., 1982; Omholt, 1988). Finally, it has been suggested that autumn pollens may contain nutrient profiles that support winter bee physiology (DeGrandi-Hoffman et al., 2018). Indeed, pollen preferences do appear to change seasonally (Bonoan et al., 2017, 2018), and newly emerged winter bees are physiologically distinct from spring/summer bees (Kunert and Crailsheim, 1988), suggesting a role of larval nutrition in winter bee development, rather than a plastic adult nurse bee state.

As environmental conditions are linked to changes in the demographic structure of the colony, it is difficult to determine which factor is the primary driver of winter bee production, and, indeed, these factors may act additively and/or synergistically (Döke et al., 2015). In this study, our goal was to assess the effect nurse load has on nurse bee physiology in summer and autumn to determine whether other seasonal factors, beyond differences in brood area, are associated with worker bee physiology. We hypothesized that there would be an additive effect of brood amount and season, suggesting that seasonal factors (other than just brood area) contribute to the summer to winter bee transition.

MATERIALS AND METHODS

Honey bee colonies

Honey bee colonies, *Apis mellifera* Linnaeus 1758, used in this study were maintained according to standard commercial practices in the apiaries at Penn State University. Our experiments were run in two seasonal rounds – summer (June–July 2021) and autumn (August–September 2020). The month before each round, we established 10-frame, single brood chamber colonies in an apiary in central Pennsylvania, USA (from splits in autumn and from packages in summer). We re-queened colonies with a newly produced, naturally mated queen derived from Italian-Carniolan stocks. In summer, we added a medium honey super above a queen excluder to each colony to prevent swarming while constraining the brood nest to a single deep hive body. In summer, the average colony size was 7.0±0.70 frames of adult bees (mean±1 s.e.m.) and in autumn, average colony size was 5.2±0.57 (Table S1) (Delaplane et al., 2013). *Varroa* mite (*Varroa destructor*) populations were managed in autumn using oxalic acid vaporization; autumn colonies received their first mite treatment 2 days prior to collection of the focal bees.

Brood manipulation

In both seasons we manipulated the amount of brood in 15 colonies (Fig. 1). Colonies were inspected and manipulated every 10 days to be higher (brood added; n=5 colonies), lower (brood removed; n=5 colonies) or serve as a control (unmanipulated, n=5) in their amount of brood. Frames were swapped between the brood-added and brood-removed colonies to create colonies with more and less brood, respectively; these pairs were randomized at each inspection/manipulation. During inspections, the area of brood at the beginning and after manipulation was noted (to the nearest quarter side of a frame), along with the presence of a laying queen, and any visual signs of disease. On average, swapping frames between the brood-added and brood-removed treatments resulted in a change of approximately a quarter of a frame of open brood in autumn, and approximately half a frame of open brood in summer (Table S1).

In autumn (2020), the total area of brood (capped pupae and open eggs/larvae) was noted and manipulated. This resulted in the brood-removed colonies growing less rapidly in adult population size than the brood-added or control colonies (Table S1). Therefore, in summer (2021) we distinguished between the area of open brood and capped brood and attempted to specifically manipulate the area of open brood because open brood is actively fed by nurse bees and emits brood pheromone. We also exchanged frames of open brood for capped brood to maintain adult populations around the same size (Table S1). Open brood area was strongly correlated with total brood area in our control (unmanipulated) summer colonies (Fig. S1). Based on this correlation, we applied a correction factor to our total brood area to estimate open brood area in autumn. Henceforth, 'brood area' refers to open brood area for summer colonies and approximated open brood area for our autumn colonies. For additional information and justification of these methods, please see Supplementary Materials and Methods.

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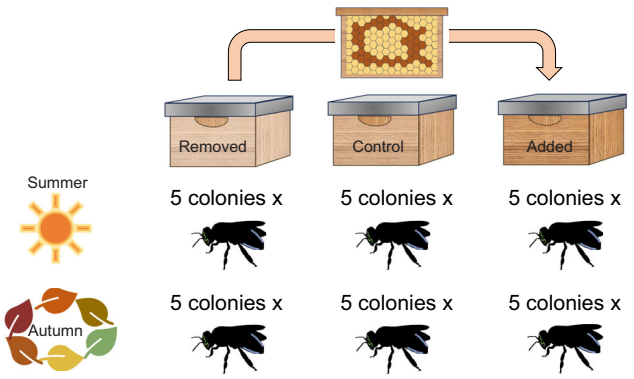

Fig. 1. Experimental design, showing colonies in the brood-removed, control and brood-added honey bee colony treatments in summer and autumn. For each treatment in this fully crossed design, 5 colonies were manipulated and n<15, 10 day old bees were sampled from each colony. These focal bees were assessed for abdomen *vitellogenin* (*vg*) expression or head mass (i.e. hypopharyngeal gland size) and fat body size. Frames of brood were swapped between brood-removed and brood-added treatments to create colonies of high and low brood in either season.

Sampling of focal 10 day old bees

Honey comb frames containing late stage capped brood, sourced from an unrelated colony in a different apiary, were emerged overnight in an incubator (34°C, 50% relative humidity) in autumn (early September 2020) and summer (early July 2021). Newly emerged bees were paint marked and introduced into the test colonies. When the introduced bees were 10 days old, <15 marked bees per colony were collected from these colonies and flash frozen on dry ice. Bees were stored at −80°C until processing.

Prior to the introduction of these bees, there were two rounds of brood manipulation, the most recent occurring 5 days prior to the introduction. Five days after the bees were introduced, colonies were again inspected and manipulated, and 5 days after the bees were sampled, the colonies were inspected for a final time.

vg expression: RNA extraction and quantitative PCR

Three colonies per season were chosen to represent the three brood manipulation treatments (one colony each). Colonies were chosen for strong differences in brood area among treatments, sufficient bees available for sampling, and colony health (no apparent signs of disease, and the presence of a laying queen). Eight sampled bees per colony were randomly chosen. Abdomens were removed and placed in ice cold RNAlater to thaw, and then internal organs were removed, leaving only the fat body tissue attached to the exoskeleton. We then extracted RNA from the eviscerated abdomens using a RNeasy Mini Kit (Qiagen, Hilden, Germany) with an RNase-Free DNase Set (Qiagen) for RNA purification. We used quantitative PCR (qPCR) to quantify the expression of *vg* in the fat bodies following previous protocols (Kocher et al., 2008; Ray et al., 2021). First, cDNA was synthesized from 200 ng of RNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ThermoFisher Scientific, Vilnius, Lithuania). cDNA was then diluted to a 1:20 concentration and qPCR was conducted on a QuantStudio 5 Real-Time PCR Instrument (Applied Biosystems, Singapore) using PowerTrack SYBR Green Master Mix (ThermoFisher Scientific). Primer details for *vg* and our two housekeeping genes, *rp-49* and *gapdh*, can be found in Table 1. qPCR samples were run in triplicate, averaged (removing Ct values>mean±0.5 as outliers), and relative *vg* expression was quantified using the $2^{-\Delta\Delta Ct}$ method with summer control colonies as the reference treatment (Schmittgen and Livak, 2008).

Fat body and head mass assessments of focal bees

The sampled, marked, 10 day old bees were assessed for fat body mass and fresh head mass in the brood-added and brood-removed treatments. Because of poor survival among marked bees introduced into control colonies, this treatment was excluded for fat body and head mass analysis. A total of 26 bees per treatment were assessed from the summer cohort, and 27 and 22 bees from the brood-removed and brood-added treatments, respectively, were assessed from the autumn cohort. An average of 7±1 bees were sampled per colony (Table 2). In some colonies, sampling was limited by marked bee survival; particularly in autumn, colonies were aggressive towards introduced marked bees, likely as a response to the threat of honey robbing. Only bees from colonies with a laying queen were included.

Fresh head mass, an approximation of hypopharyngeal gland mass and acini size (Hrassnigg and Crailsheim, 1998; Hendriksma et al., 2019), was quantified using a microbalance (VWR-205TC Balance, Philadelphia, PA, USA) with readability to 0.01 mg. Fat body mass was approximated following methods modified from Fischer and Grozinger (2008). Briefly, abdomens from the same bees were eviscerated, lyophilized for 4 h, and then measured for mass using the same microbalance. Dried abdomens were then submerged in 1 ml of 2:1 chloroform:methanol solution overnight to dissolve the fat body, after which the solution was pipetted off, and any residual solution was allowed to evaporate overnight. Abdomens were then remeasured to find their mass, and the mass of the fat body was calculated as the change in mass of the abdomens before and after dissolution and removal of the fat body, divided by the second mass to correct for variation in abdomen exoskeleton size.

Statistical analysis

All statistical analysis was completed in R (v.4.2.0; http://www.R-project.org/). We first assessed the effectiveness of our brood manipulation treatments on utilized colonies by regressing brood area [log(x+1) transformed] from all three manipulations with season, treatment group and their interaction. We additionally included random effects of colony and inspection date, using the lme4 package (Bates et al., 2015). We further decomposed this relationship by stratifying our model by season, log-transforming autumn brood area but not summer brood area. We similarly assessed differences in open brood area when excluding the control

Table 1. Primer information

Target gene	Gene description	Primer sequence (forward and reverse)	T_m (°C)	Reference
vg	Vitellogenin	TTGACCAAGACAAGCGGAACT	57	Kocher et al., 2008
		AAGGTTCGAATTAACGATGAAAGC	54	
gapdh	Glyceraldehyde 3-phosphate dehydrogenase	GCTGGTTTCATCGATGGTTT	54	Huang et al., 2012
		ACGATTTCGACCACCGTAAC	55	
rp-49	Ribosomal protein 49	AAGTTCATTCGTCACCAGAG	52	Grozinger et al., 2003; de Miranda and Fries, 2008
		CTTCCAGTTCCTTGACATTATG	51	

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Table 2. Number of 10 day old focal bees collected and assessed per colony, per brood manipulation treatment within each season

Season	Treatment	Colony	Bees (*n*)
Summer	Brood added	1	9
Summer	Brood added	2	4
Summer	Brood added	3	4
Summer	Brood added	4	9
Summer	Brood removed	5	4
Summer	Brood removed	6	4
Summer	Brood removed	7	9
Summer	Brood removed	8	9
Autumn	Brood added	9	10
Autumn	Brood added	10	10
Autumn	Brood added	11	2
Autumn	Brood removed	12	6
Autumn	Brood removed	13	3
Autumn	Brood removed	14	13

colonies, which had a low sample size because they were only utilized for *vg* comparisons. We then used linear models to describe the relationship between each of our dependent variables (relative *vg* expression, head mass and fat body mass) with our predictor variables of season and brood area. For relative *vg* expression [log fold-change, i.e. $\log_{10}(2^{-\Delta\Delta Ct})$], we used a simple linear model to assess the effect of season and brood area, with and without their interaction. We tested both the average brood area across all manipulations and the brood area resulting from manipulation when marked bees were 5 days old (Table 3). Over time, colonies may adjust the area of brood by cannibalizing brood they are unable to support (Schmickl and Crailsheim, 2001). Therefore, we felt the day-5 post-manipulation measurement of brood may more accurately reflect the environment to which the young bees were exposed to during a critical period of their life. Because brood area was correlated with season, we used Akaike information criterion values corrected for small sample size (AICc) to compare these models using the bbmle package (https://CRAN.R-project.org/package=bbmle). For head mass and fat body mass, we similarly used linear regression models, this time with a log-normal error distribution and a random effect of host colony using the lme4 package (Bates et al., 2015), in addition to season, average brood area across all inspections, and their interaction as fixed effects. We likewise compared models using AICc values and maximum likelihood estimates (Table 3).

Table 3. Model comparisons based on ΔAICc values, degrees of freedom and model weights for each set of models

Model	Predictors	ΔAICc	d.f.	Model weights
Relative *vg* expression (mean brood)	Season	0.0	3	0.57
	Season+Brood area	2.4	4	0.17
	Season+Brood area+ Season:Brood area	2.5	5	0.16
	Brood area	3.6	3	0.09
	Null (intercept only)	11.8	2	0.00
Relative *vg* expression (manipulated brood)	Season	0.0	3	0.56
	Season+Brood area	1.0	4	0.34
	Season+Brood area+ Season:Brood area	3.5	5	0.10
	Null (intercept only)	11.8	2	0.00
	Brood area	13.6	3	0.00
Fat body	Season	0.0	5	0.50
	Season+Brood area	1.6	6	0.23
	Season+Brood area+ Season:Brood area	1.6	7	0.22
	Null (intercept only)	5.4	4	0.03
	Brood area	5.9	5	0.03
Head mass	Season+Brood area	0.0	6	0.52
	Season	1.5	5	0.24
	Season+Brood area+ Season:Brood area	1.7	7	0.23
	Brood area	7.1	5	0.02
	Null (intercept only)	23.8	4	0.00

Four sets of models were compared: relative *vg* gene expression (using mean open brood area across all manipulations as well as the area of open brood at the most recent manipulation), fat body mass and head mass as outcome variables. AICc, Akaike information criterion, corrected for small sample size; d.f., degrees of freedom.

RESULTS

Brood area differed by season ($F_{1,9.63}$=5.07, P=0.05), with higher brood area in summer than in autumn. Brood manipulation treatment on its own was not significant ($F_{2,9.54}$=1.66, P=0.24). However, the interaction between season and treatment was significant ($F_{2,9.54}$=5.55, P=0.03), with the brood-removed treatment having a significantly lower brood area than the brood-added treatment ($t_{6.00}$=−4.36, P<0.01) in autumn ($F_{2,6}$=10, P=0.01). In summer, there were no significant treatment differences ($F_{2,4.72}$=0.48, P=0.65). When excluding the control colonies, we observed the same trends, whereby brood area differed seasonally ($F_{1,7.72}$=6.48, P=0.04) but not between treatments ($F_{1,9.43}$=2.67, P=0.13), and treatment was only effective in autumn ($F_{1,6}$=19.05, P<0.01), but not summer ($F_{1,4.81}$=0.93, P=0.38).

The season-only model was the most parsimonious model for describing relative *vg* expression in focal 10 day old bees (Table 3), both for the average brood models and those using brood area from the manipulation 5 days before nurse bees were collected (Fig. 2). Relative *vg* expression was highest among summer bees [95%

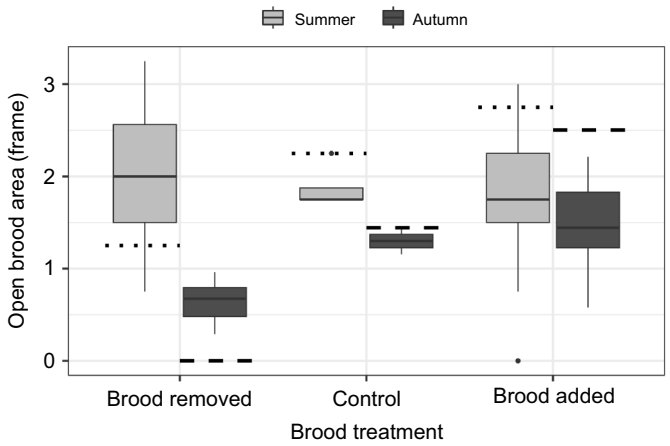

Fig. 2. Differences in open brood area among the different honey bee colony brood manipulation treatments in summer and autumn. Box plots show median (horizontal line inside boxes), first and third quartiles (box), and 1.5× interquartile range (whiskers). Dotted/dashed horizontal lines indicate the amount of open brood after manipulations, 5 days before 10 day old bees were collected. Within each season, 15 colonies were manipulated (5 per treatment). Focal bees from 9 colonies (4 brood-added, 4 brood-removed, 1 control colony) were utilized in this study (control colonies were only used for *vg* comparisons). Each colony was inspected 4 times. There were not significant treatment-level differences in open brood area among these colonies based on analysis of variance (see Materials and Methods for model details), both when including ($F_{2,9.54}$=1.66, P=0.24) and when excluding ($F_{1,9.43}$=2.67, P=0.13) the control treatment. There were, however, seasonal differences (full model: $F_{1,9.63}$=5.07, P=0.05; without control: $F_{1,7.72}$=6.48, P=0.04), as well as treatment differences within autumn (full model: $F_{2,6}$=10, P=0.01; without control: $F_{1,6}$=19.05, P<0.01).

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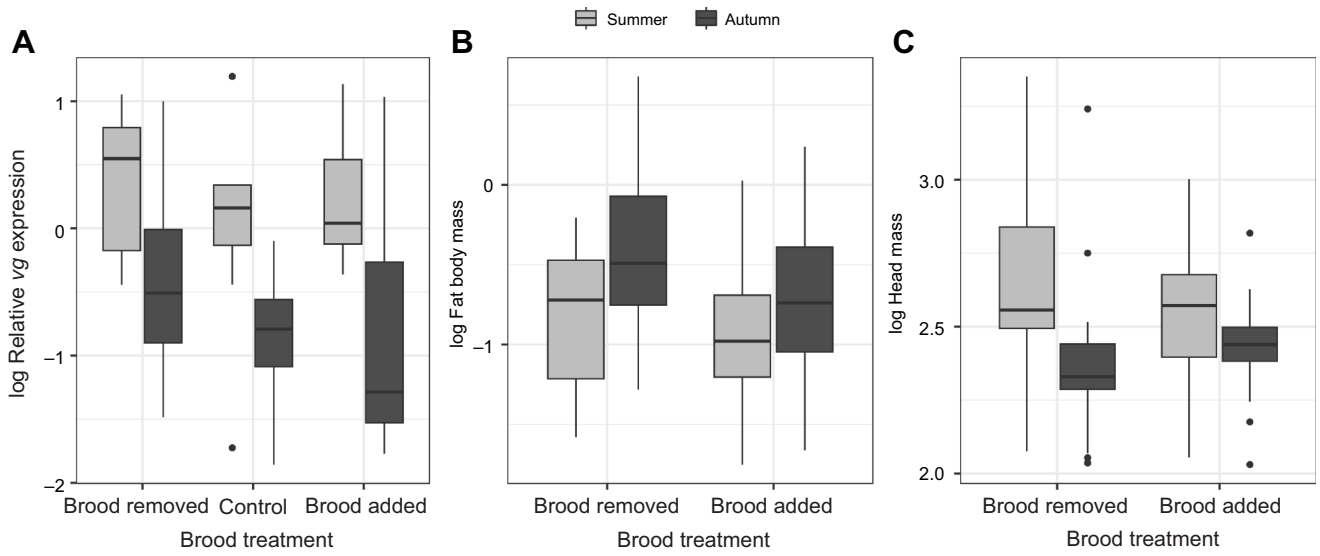

Fig. 3. Differences in relative *vg* expression, fat body mass and head mass among nurse bees from the different honey bee colony brood manipulation treatments in summer and autumn. Six colonies (one colony per treatment, per season) were assessed for *vg* expression ($2^{-\Delta\Delta Ct}$; A), with eight focal 10 day old nurse bees assessed per colony (one bee excluded from summer brood-removed treatment, autumn brood-added treatment, and autumn brood-removed treatment based on exclusion criteria, see Materials and Methods for details). Sample sizes for fat body mass (mg; B) and head mass (mg; C) are shown in Table 2. Generalized linear (mixed) models and Akaike information criterion were used to compare brood treatments, seasons and their interaction for describing nurse bee physiology. See Table 3 for model comparisons.

confidence interval (CI)=−1.31–−0.43]. While there was a trend in both seasons for a negative correlation between relative *vg* expression and brood area (Fig. 3A), and there was some evidence for a contribution of brood area in the models which used recently manipulated brood area (Table 3), this relationship was somewhat uncertain (CI=−0.41–0.11). Brood area did not sufficiently contribute to the model (based on ΔAICc>2) (Burnham and Anderson, 2007) in the models which used average brood area.

Fat body mass was similarly best explained by season alone; the next best model, which included both season and brood area, was not sufficiently different from the season-only model (ΔAICc=1.57). Fat bodies were smaller among focal bees collected in summer than in those collected in autumn (CI=−0.50–−0.09) (Fig. 3B).

The best model for describing head mass included both brood area and season; however, this model was likewise not sufficiently different from the season-only model (ΔAICc=1.55) or the model which included season, brood area and their interaction (ΔAICc=1.65). The brood and season model suggests that summer nurse bees have heavier heads than autumn bees (CI=0.03–0.12) and that brood area is positively correlated with head mass (CI=0.00–0.07) (Fig. 3C). The random effect of colony identity caused a singular model fit for the head mass models. Using total brood area (rather than open brood area) yielded very similar results overall (Table S2). Published data are available in Tables S3–S5.

DISCUSSION

While there were seasonal shifts in the abundance of brood within colonies, our data suggest that most of the variation in young worker bee physiology in summer versus autumn is attributable to seasonal factors, rather than a changing brood environment alone. Ten day old focal bees in autumn had larger fat bodies, but lower relative *vg* expression, and lighter heads (suggesting smaller hypopharyngeal glands; Hrassnigg and Crailsheim, 1998) than summer bees. In some cases, particularly for head mass, brood area modified these seasonal trends, with bees exposed to more brood having larger provisioning glands. These findings suggest that additional seasonal factors beyond brood environment could contribute to bee physiology, and perhaps explain the transition to a winter bee state.

Season – not brood area – was the most parsimonious physiological predictor for both *vg* expression and fat body size. In many cases, the addition of brood area as a predictor did not reduce the performance of the model, but it also did not substantially improve it. This finding runs somewhat contradictory to the theory that nurse load (which is correlated with brood area) is the primary driver of winter bee production (Amdam et al., 2009b). Rather, it seems that other seasonal factors likely play an important role in adult bee physiology. While we did observe a negative correlation between brood area and both *vg* expression and fat body size, as we would expect based on previous literature (Maurizio and Hodges, 1950; Eishchen et al., 1982; Omholt, 1988; Amdam et al., 2009b; Smedal et al., 2009), this trend was somewhat uncertain (95% CI contained zero) and the biological effect was small compared with the effect associated with season. This highlights the importance of examining these trends at different times of the year to understand the relative effect and context-specific nature of each of these factors.

Notably, we observed opposite seasonal effects for relative *vg* expression and fat body size. While fat bodies were larger in autumn, which would track with a more winter-like physiology (Shehata et al., 1981; Kunert and Crailsheim, 1988; Döke et al., 2015; Knoll et al., 2020), *vg* expression was higher among summer bees. Because the abdominal fat body is the primary site of *vg* expression (Snodgrass, 1956; Corona et al., 2007; Amdam et al., 2012), we would have expected these two biomarkers to yield similar results. Lower *vg* expression in autumn than in summer is particularly remarkable given previous studies that have reported higher Vg titers in winter bees than in summer bees (Fluri et al., 1982; Fluri and Bogdanov, 1987a) and that other seasonal environmental factors, such as temperature and photoperiod, may also indirectly increase Vg (Fluri and Bogdanov, 1987b; Huang and Robinson, 1995; Döke et al., 2015). Still, similar work by Steinmann et al. (2015) found that *vg* expression was lower in autumn (September) than in summer

(June) when bees were <30 days old, and only increased later in autumn (October/ November). This is consistent with our findings, and it suggests that we may have detected an increase in *vg* expression had we continued to monitor these populations later into the winter. Thus, it is possible that higher Vg reported among winter bees in previous studies relates to higher protein storage (but not necessarily synthesis).

Varroa mite levels, which are typically higher in autumn (DeGrandi-Hoffman et al., 2016; Jack et al., 2023), may also have contributed to differences in *vg* expression (Dainat et al., 2012; Steinmann et al., 2015; Alaux et al., 2017). Previous studies have shown that *Varroa* infestation is associated with decreased *vg* expression at the colony level (Dainat et al., 2012; Alaux et al., 2017). While we did not assess *Varroa* populations, we observed the lowest *vg* expression among our brood-added colonies in autumn. Because *Varroa* reproduce in capped brood cells (Donzé et al., 1998), increasing the amount of brood in the brood-added colonies, particularly in autumn, may have also increased *Varroa* populations within the colony and decreased *vg* expression. However, work by Steinmann et al. (2015) showed that *vg* expression can also increase as an immune response to *Varroa*-associated deformed wing virus (DWV) in summer, but that there is no relationship between DWV levels and *vg* expression in autumn. We also cannot disregard the possible effects of our *Varroa* treatment on autumn colonies. While oxalic acid is a natural 'soft' chemical treatment for *Varroa* management (Jack and Ellis, 2021) that has been shown to be >90% effective at controlling *Varroa* in colonies with brood (Rademacher and Harz, 2006) and is generally safe for bees when used at the colony level (Rademacher and Harz, 2006), some work suggests that it could have sublethal effects on adult bees and could damage brood (Schneider et al., 2012; Rademacher et al., 2017).

Another possible explanation for higher *vg* expression in summer is the availability of better nutrition in the summer, as *vg* is a biomarker of nutrition and indicative of well-fed bees (Alaux et al., 2011; 2017). Work by DeGrandi-Hoffman et al. (2018) shows that autumn bees upregulate *vg* expression when fed spring pollen, as opposed to autumn pollen, indicating the nutritional value of spring pollen and its capacity to affect *vg* expression. Furthermore, nutritional resource limitation in autumn has been linked to the summer-to-winter bee transition, though the authors suggest this works through indirect effects on brood area and demonstrate that the bees that emerge after this reduction become the long-lived winter bees (Mattila and Otis, 2007). Because we introduced adult bees from an unrelated colony, the focal bees may have missed key colony environment signals during their larval stage. We note that our *vg* expression analysis was only conducted on one colony per treatment (n=8 bees per colony). This was done to minimize colony-level variation in gene expression but may limit the broader applicability of our results. Future more temporally resolved studies across a greater number of colonies may be necessary to understand the direct and indirect effects of pollen nutrition on the winter bee transition.

Head mass was the only physiology metric for which season alone was not the best predictor; we found evidence for heavier heads among summer bees than autumn bees and among colonies with more brood. The effect of brood can be easily explained – brood and/ or brood pheromone is necessary for hypopharyngeal gland growth and development (Huang and Otis, 1989; Mohammedi et al., 1996; Hrassnigg and Crailsheim, 1998; Traynor et al., 2017), and exposure to brood pheromone increases hypopharyngeal gland protein content (Pankiw, 2004; Sagili and Pankiw, 2009). However, the seasonal finding runs contradictory to our expectations. Many studies describe enlarged hypopharyngeal glands among winter bees (Fluri et al., 1982; Moritz and Crailsheim, 1987), with head glands functioning for nutrient storage in the winter rather than nutrient provisioning as they do in the summer (Brouwers, 1983). In a very similar study to ours, Moritz and Crailsheim (1987) describe hypopharyngeal gland depletion among July bees (>8 days old), while September bees maintained large hypopharyngeal glands from 8 to 20 days old. Therefore, we expected to observe larger head glands among our September 10 day old focal bees compared with July bees, possibly reflecting bees making the physiological transition to winter. Like *vg* expression, however, superior summer nutrition may explain the seasonal trends we observed in head gland size; DeGrandi-Hoffman et al. (2018) describe larger hypopharyngeal glands among summer bees fed summer pollen than in those fed autumn pollen.

By using full-size, free-flying colonies, we hoped to capture field-realistic dynamics underlying the transition from summer to winter physiology in honey bees. Nevertheless, this system introduces several potentially confounding factors that may have affected the conclusions of our study. Most notably, our brood manipulation treatments were not effective in summer, though they were effective in autumn. This was likely the result of fast-growing colonies that are able to rear abundant brood during the summer growing season, as opposed to autumn when brood production naturally slows (Winston, 1987). Despite our treatment groups being somewhat ineffective, we were still able to capture a range of brood area conditions across colonies between the seasons. This allowed us to parse the effects of season separate from the effect of brood area. It is possible that with a more extreme reduction or addition of brood – as in previous studies that examined entirely broodless colonies (Huang and Otis, 1989; Mohammedi et al., 1996) – we may have seen even more pronounced effects. There could also have been confounding effects of the population of young bees in the colonies, which would contribute to each nurses' relative load and could influence the winter bee transition by 'pushing' middle-aged bees from the colony (Amdam et al., 2009b; Johnson, 2010). While we shook nurse bees off frames before swapping them to reduce this effect, abundant emerging brood in our brood-added colony could have increased the population of young bees. This effect would be most likely in autumn 2020, when we did notice an increase in adult population size in the brood-added treatment. However, many of the treatment effects of brood area appear to be more pronounced in autumn (Fig. 3), suggesting this dynamic is of little concern to our broader conclusions. Finally, we did not assess focal bee longevity, which would have been a good indicator of the winter bee phenotype. We do, however, present compelling evidence of other winter bee biomarkers, which we believe provides strong evidence for the relative effect of brood area versus season on bee seasonal physiology. We note that the poor survival described for the control colonies (particularly in autumn), which prevented us from analyzing this treatment group for fat body and head mass, was not related to longevity. Rather, we observed aggression towards introduced bees and saw marked bee body parts disposed of at the hive entrance. This treatment-specific aggression may relate to these colonies being less accustomed to disturbance (Rittschof, 2017) and/or more genetically heterogeneous in their populations because frames of brood were not swapped in this treatment. While not directly related to the aims of this study, future work could examine the role of colony genetic diversity on social defensiveness [see previous work by Hunt et al. (2003) and Rittschof et al. (2015) for evidence of social environment on individual aggression].

In this study, we assessed the effect of brood area on nurse bee physiology in both summer and autumn to better understand the relative contribution of brood area to the summer-to-winter bee transition. We found that season played a more significant role in many nurse/winter bee physiological markers relative to colony social demographic structure; namely, brood area. Our findings suggest that environmental seasonal factors, including photoperiod, temperature and forage availability, play a role in the transition of bees to a winter-like state. It remains to be determined whether the effect is direct, or indirect through influencing foraging behavior or diet. This finding has important implications for our understanding of basic honey bee biology and the management of honey bee colonies for the winter. Winter is the time of greatest honey bee colony loss (vanEngelsdorp and Meixner, 2010), so a better understanding of winter bee biology could help inform better management practices to improve colony survival (Döke et al., 2015).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.M.Q., C.M.G.; Methodology: G.M.Q., C.M.G.; Software: G.M.Q.; Validation: C.M.G.; Formal analysis: G.M.Q.; Investigation: G.M.Q.; Resources: C.M.G.; Data curation: G.M.Q.; Writing - original draft: G.M.Q.; Writing - review & editing: C.M.G.; Visualization: G.M.Q.; Supervision: C.M.G.; Project administration: G.M.Q., C.M.G.; Funding acquisition: G.M.Q., C.M.G.

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Data availability
All relevant data can be found within the article and its supplementary information.

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