The Company of Biologists

RESEARCH ARTICLE

Shift in worker physiology and gene expression pattern from reproductive to diapause-like with colony age in the bumble bee *Bombus impatiens*

Erin D. Treanore, Jacklyn M. Kiner, Mackenzie E. Kerner and Etya Amsalem*

ABSTRACT

Insects maximize their fitness by exhibiting predictable and adaptive seasonal patterns in response to changing environmental conditions. These seasonal patterns are often expressed even when insects are kept in captivity, suggesting they are functionally and evolutionarily important. In this study, we examined whether workers of the eusocial bumble bee *Bombus impatiens* maintained a seasonal signature when kept in captivity. We used an integrative approach and compared worker egg laying, ovarian activation, body size and mass, lipid content in the fat body, cold tolerance and expression of genes related to cold tolerance, metabolism and stress throughout colony development. We found that bumble bee worker physiology and gene expression patterns shift from reproductive-like to diapause-like as the colony ages. Workers eclosing early in the colony cycle had increased egg laying and ovarian activation, and reduced cold tolerance, body size, mass and lipid content in the fat body, in line with a reproductive-like profile, while late-eclosing workers exhibited the opposite characteristics. Furthermore, expression patterns of genes associated with reproduction and diapause differed between early- and late-eclosing workers, partially following the physiological patterns. We suggest that a seasonal signature, innate to individual workers, the queen or the colony, is used by workers as a social cue determining the phenology of the colony and discuss possible implications for understanding reproductive division of labor in bumble bee colonies and the evolutionary divergence of female castes in the genus *Bombus*.

KEY WORDS: Caste, Cold tolerance, Gene expression, Reproduction, Seasonality, Social insects

INTRODUCTION

Insects maximize their fitness by exhibiting predictable and adaptive seasonal patterns in their physiology. Examples of the two most extensively studied phenomena are seasonal migratory movement and preparation for the winter diapause. These phenomena allow an organism to survive unsuitable environmental conditions either through movement (migration) or avoidance (diapause) and are often characterized by shifts in metabolism and reproduction, and increased tolerance to environmental stressors (Koštál, 2006; Koštál and Denlinger, 2011; Ramenofsky and Wingfield, 2007). These

Department of Entomology, Center for Chemical Ecology, Center for Pollinator Research, Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA 16802, USA.

*Author for correspondence (eua6@psu.edu)

E.D.T., 0000-0001-9555-0395; E.A., 0000-0001-7935-049X

adaptations evolved in response to changing environmental conditions, such as natural cycles of temperature, photoperiod, humidity and resource availability, leading to changes in insect abundance, mating, reproductive and dispersal habits as the season progresses (Denlinger, 2002; Gotthard, 2001). For example, decreased photoperiod and fluctuating temperatures can induce a reproductive diapause in the monarch butterfly (*Danaus plexippus*), and reduced photoperiod leads to increased lipid accumulation in bean bugs (*Riptortus clavatus*) (Goehring and Oberhauser, 2002; Morita et al., 1999). Similarly, reduced resource availability induces dispersal by insects to areas more suitable for nest foundation and reproduction (Benton and Bowler, 2012), and reduced temperatures induce migratory behavior in butterflies and dragonflies (Chapman et al., 2015; Mikkola, 2003; Wikelski et al., 2006), overall allowing organisms to avoid otherwise unsuitable environmental conditions and to synchronize their life history with other members of the population.

Insects often exhibit cyclic patterns associated with seasonality even when they are held in controlled conditions, provided with unlimited food or kept in captivity (Blake, 1960; Mao and Henderson, 2007; Miyazaki et al., 2014; Nisimura and Numata, 2003). Such a seasonal signature that is maintained regardless of external conditions suggests functional importance, and highlights aspects related to the evolutionary history of species.

Social insects are a fascinating model in which to study these seasonal signatures in the wild and in the laboratory as seasonal changes may evolve to be used as social cues determining social behavior and the phenology of the colony. For example, honey bee (*Apis mellifera*) workers go through physiological changes throughout the season. Workers eclosing in the spring have reduced ovarian activation as compared with their summer and autumn eclosing sisters, likely a result of both reduced larval nutrition and cooler springtime temperatures (Hoover et al., 2006). Interestingly, honey bee workers exhibit these seasonal patterns even when reared in the laboratory under controlled conditions, with spring and autumn workers possessing different nutritional requirements, aligning with the colony's activities and needs across its yearly cycle (DeGrandi-Hoffman et al., 2018). Likewise, bumble bee workers (*Bombus terrestris*) eclosing in older laboratory colonies (comparable to late season colonies) show increased levels of constitutive immune defenses as compared with younger colonies (Moret and Schmid-Hempel, 2009). Ants provide several other fascinating examples: *Formica* ants reared in the laboratory under constant temperatures show distinct developmental patterns (Kipyatkov, 1993), and fire ant (*Solenopsis invicta*) workers from colonies collected in the summer and autumn and maintained under laboratory conditions show distinct physiological phenotypes, with autumn ants accumulating significantly more lipids (Cook et al., 2016). However, the extent to which the seasonal and the social cues

1

are conflated and whether social cues are tied to innate seasonal signatures, that are independent from the surrounding conditions, is unknown.

In this study, we examined whether workers of the eusocial bumble bee *Bombus impatiens* Cresson 1863 maintained a seasonal signature when kept in captivity. Bumble bees provide an excellent and tractable model system to examine this question for two reasons relating to their eusocial life cycle and their evolutionary history. First, bumble bees are annual eusocial species that go through a predictable life history trajectory as the colony ages. The newly mated queens disperse by late summer and enter a winter diapause lasting 6–9 months during which they are metabolically suppressed. Prior to diapause, queens will mate and accumulate fat reserves such as lipids, and their ovaries will remain inactive until the end of diapause. The transition to diapause is further characterized by a shift in hormone levels (e.g. juvenile hormone, JH) and gene expression associated with metabolism, stress and reproduction (Amsalem et al., 2015a). A single queen will then found a colony in early spring and the worker force is then progressively built up to reach a size of several hundred workers by late summer (Amsalem et al., 2015b). The life cycle of the colony can be divided into two major social phases starting from the eclosion of the first worker (Duchateau and Velthuis, 1988). During the first phase (the pre-competition phase), which lasts approximately 4–5 weeks, the queen is the sole producer of eggs and workers function as helpers, whereas during the second phase (the competition phase), workers may exhibit aggression and attempt to lay eggs, sexuals are produced and the social organization breaks down. While the triggers for the competition phase and the production of sexuals are not fully clear, it was suggested that workers modify their reproduction in response to changes in the queen (Almond et al., 2019; Amsalem et al., 2015a), the brood (Starkey et al., 2019a,b) and the demography of the colony (i.e. the number of workers) (Amsalem and Hefetz, 2011; Bloch, 1999; Van Doorn and Heringa, 1986). While earlier studies considered workers as a 'clean slate' (Amsalem and Hefetz, 2010), proposing that workers are morphologically, physiologically and behaviorally identical regardless of the time they eclosed during the life cycle, later studies show that workers eclosing at different times vary in intrinsic quality (Blacher et al., 2017; Couvillon et al., 2010; Shpigler et al., 2013). However, these investigations were limited to qualities such as body size and reproduction and did not explore further characteristics. Furthermore, it is unknown whether these changes are driven by colony- or individual-level shifts in worker physiology.

Second, all bumble bees evolved from a eusocial species where all females were likely morphologically identical into a species where only large, fecund queens, but not workers, undergo a winter diapause (Cameron et al., 2007; Danforth et al., 2013; Hines, 2008; Wheeler, 1986). Bumble bee workers are, on average, 3 times smaller than the queens, do not mate, remain largely sterile and lack the physiological capacity to accumulate the fat body reserves necessary to survive the winter diapause (Amsalem et al., 2015b; Couvillon and Dornhaus, 2010). Thus, the differentiation of female castes could have been influenced by seasonal pressures shaping female physiology, an idea originally proposed for wasps where caste differences were suggested to be driven by nutritionally sensitive ancestral genetic and physiological signatures that bifurcate during larval development in seasonal environments (the diapause ground plan hypothesis, DGPH) (Hunt, 2006). This is further supported by the most recent reconstruction of the *Bombus* phylogeny suggesting that bumble bees were subjected to an extensive period of cooling and are assumed to have radiated during the Miocene (Hines, 2008; Prokop et al., 2017), an epoch associated

with gradual cooling that could have potentially shaped bumble bee social evolution. Overall, the maintenance and expression of a seasonal signature in females (both queens versus workers and within workers across the season) may generate predictions about the evolutionary trajectory of bumble bee caste divergence as a function of the environmental conditions.

To examine whether workers kept in captivity maintain a seasonal signature that corresponds to the age of the colony, we compared worker physiology and gene expression patterns throughout colony development. We hypothesized that bumble bee worker physiology changes as the colony ages and discuss different explanations underlying the observed changes. Specifically, we discuss four explanations for the physiological differences found in workers throughout the life cycle. (1) Worker physiology changes over time regardless of external conditions as they carry a signature indicative of their evolutionary history. Thus, the physiological signature is innate to workers. Here, we discuss the DGPH (Hunt, 2006), suggesting that social insect castes (sterile, short-lived workers versus fecund long-lived queens), diverged as a result of seasonal pressures. (2) Worker physiology is influenced by the social environment. Thus, the observed physiological changes are determined by the colony demography, can be controlled by changing the social environment and are not innate. (3) Workers evolved to use seasonal cues as social cues determining female reproduction. Thus, the observed physiological changes are innate to the colony. (4) Worker physiology is dictated by the queen's age at the time of egg laying. Thus, the observed changes are innate to the queen.

To address the main question, we used an integrative approach and compared worker egg laying, ovarian activation, body size and mass, lipid content in the fat body, cold tolerance and expression of genes related to cold tolerance, metabolism and stress throughout colony development in colonies reared under controlled conditions. Genes were selected based on their role in physiological processes associated with seasonality, mainly diapause and reproduction. We examined the expression levels of (1) *foxo*: a forkhead transcriptional factor in the insulin pathway also shown to be downregulated during diapause (Sim and Denlinger, 2008); (2) *menin*: a regulator of the general stress response previously found to be upregulated during diapause in *B. terrestris* queens (Amsalem et al., 2015a); (3) *hsp70*: a heat shock protein that is produced in response to cold in insects (Rinehart et al., 2007) and based on previous studies is expected to be upregulated during diapause; (4) *pepck*: a metabolic enzyme involved in gluconeogenesis (Yang et al., 2009) that is expected to be downregulated during diapause; (5) *hex1*: hexamerin storage protein that is produced in preparation for diapause (Hunt et al., 2007) and is expected to be upregulated during diapause; (6) *mfe*: the final enzyme in the synthesis of JH, the main gonadotropin in insects (Daimon and Shinoda, 2013) that is predicted to be downregulated during diapause and upregulated in reproductives; and (7) *vitellogenin* (*vg*): the major egg yolk protein that female insects invest in the ovaries (Tufail et al., 2014) that is predicted to be downregulated during diapause and upregulated in reproductives.

MATERIALS AND METHODS
Bumble bee rearing

Bombus impatiens colonies (*n*=13; Table S1 includes information on the development and size of colonies used in this study, as well as the number of workers sampled from different colonies for each of the different analyses described below) were obtained from BioBest at the age of 2–3 weeks after the eclosion of the first worker or reared in the laboratory from gynes produced in BioBest colonies using a standard CO_2 treatment protocol. Briefly, newly mated

queens were placed in a closed (but not sealed) plastic cage. A steady stream of pure CO_2 was applied to the cage through a designated hole for 1 min. Under these conditions, CO_2 reaches 100% within approximately 20 s (the time it takes bees to lose mobility) and remains this way for as long as the stream lasts. Queens were then kept in the cage for an additional 30 min until they revived (Amsalem and Grozinger, 2017). Two more colonies were reared from queens caught in the wild in the spring of 2018 in Newport and Landisville, PA, USA. All colonies were kept in constant darkness, 60% humidity and 28–30°C, and manipulated under red light, which bees are unable to see. Bees were not allowed to leave the colony, and pollen and 60% sucrose solution were provided *ad libitum*. Bumble bees produce sexuals using a 'bang' strategy: an abrupt switch from the exclusive production of workers to the exclusive production of sexuals (Duchateau and Velthuis, 1988). Thus, in all experiments, workers were collected from queenright colonies (i.e. queen is present) throughout the natural development of the colonies until sexuals appeared. Workers eclosing early and late in the colony cycle (measured from the eclosion of the first worker) were considered early- and late-season workers, respectively.

Bumble bee sampling

Sampling of workers from the parental colonies began approximately 2 weeks after eclosion of the first worker and continued until workers were no longer produced; colonies were queenright for the entire sampling period. Colonies were terminated when only males or gynes were exclusively produced for five consecutive days. Worker age was differentially controlled for in the different experiments. Age was not controlled for in the experiments examining ovary size, body mass and size, was partially documented in the cold tolerance experiment (in 2 out of 6 colonies) in order to verify our method of sampling (see below), and was controlled for in egg-laying assays (all workers were 10 days old), lipid content experiment (workers were 2–11 days old) and gene expression assays (all workers were 3 days old). In experiments where age was partially or not controlled for, sampling occurred at a high rate ensuring a constant turnover of

individual workers in the colony and a colony size of 20–30 workers at all times. To verify that our sampling method maintained a constant turnover of workers in the colony, we individually marked all workers (n=975) upon eclosion from a subset of the colonies used in our analyses and found that 92% of workers were sampled within a week of eclosion. We collected information about colony life span, the date of production of the first sexuals (males and gynes), the number of workers each colony produced, and which colonies were used in each analysis (Table S1). Overall, two colonies were used to examine worker body mass, size and ovary activation (n=472), four colonies were used to examine egg laying in same-age workers (n=422), four colonies were used to examine fat body lipids (n=90), and six colonies were used to examine cold tolerance (n=2417) and fat body gene expression (n=17).

Ovary activation, body mass and size

To examine the reproductive potential of workers across the colony's development (Table 1), groups of eight randomly sampled workers were removed from their parental queenright colonies every 2–4 days throughout the colony's natural life cycle (weeks 3–13) and kept at −20°C until analysis. Age was not controlled for; however, sampling was done at a high rate (the two colonies were sampled 59 times, 8 workers at a time, during a period of 10 weeks), ensuring a constant turnover of individual workers in the colony and a colony size of 20–30 workers at any time point. The length of the terminal oocyte in the three largest ovarioles was measured with a scaled ocular. *Bombus impatiens* females possess four ovarioles per ovary and at least one ovariole per ovary was measured. Mean terminal oocyte length for each bee was used as an index of ovarian activation. The same workers were measured for their wet body mass using an electronic scale and for their body size by measuring the width of the head as in Amsalem and Hefetz (2010).

Egg laying

To examine the physiological aptitude of queenless workers (i.e. queen is absent) to lay eggs, we counted the number of eggs laid by same-age (10-day-old) workers sampled from four colonies throughout the entire life cycle (weeks 2–10). Two newly eclosed

Table 1. Examined variables and findings in this study

Variable	Function and predictions	Results of this study
Physiology		
Oocyte size	Females with a higher reproductive potential exhibit larger oocyte size	Smaller oocyte size in late-eclosing workers
Egg laying	Females with higher reproductive output lay more eggs	Lower number of eggs laid in late-eclosing workers
Cold tolerance	Females exhibit higher cold tolerance in preparation to diapause	Higher cold tolerance in late-eclosing workers
Body size	Females more adapted to diapause conditions are larger	Larger body size in late-eclosing workers
Body mass	Females more adapted to diapause conditions are heavier	Larger body mass in late-eclosing workers
Fat body lipid	Females more adapted to diapause conditions have higher fat storage	Higher fat storage in late-eclosing workers
Gene expression		
vg	Positively associated with female fertility – reproductive females show higher *vg* expression compared with non-reproductives	No differences
mfe	Positively associated with female fertility – reproductive females have higher juvenile hormone levels compared with non-reproductives	Higher in early-eclosing workers
hex1	Encodes storage proteins that accumulate in individuals during diapause	Higher in late-eclosing workers
hsp70	Encodes a heat shock protein; higher expression in individuals during diapause	No differences
menin	A regulator of heat shock proteins and a general stress response; higher expression in individuals during diapause	Higher in early-eclosing workers
foxo	A transcriptional factor in the insulin pathway; lower expression during diapause	No differences
pepck	Encodes a metabolic enzyme used during gluconeogenesis; reduced gluconeogenesis and a conservation of energy stores is typical in individuals during diapause	Higher in early-eclosing workers

workers of similar size were removed from their parental colony and placed into a cage (11 cm diameter×7 cm height) where they were provided with pollen and 60% sucrose syrup *ad libitum*. Cages were inspected for the total number of eggs laid by workers at the end of the 10 day period. Post-analysis, the data were divided to three time points (earlier than week 5, weeks 6–8 and week 9 or later), based on the division observed in the ovary experiment where these groups clustered together. These time points also matched the life cycle phases of *B. impatiens* (pre-competition, post-competition and sexual production).

Cold tolerance
To examine the cold tolerance of workers across the colony's development, we used a modified protocol from Karsai and Hunt (2002). Groups of 10 workers were randomly sampled from their parental colony throughout the entire colony cycle (weeks 3–13). In two colonies, all workers were individually marked daily upon eclosion and put back into the colony, making up a total of 40% of all workers analyzed for cold tolerance. Workers that had their age documented were 2–21 days old at the time of sampling and 92% of them were sampled within 7 days of eclosion. Workers were placed in a 28×22×5 cm cardboard box in a refrigerator mimicking diapause-like conditions (70% humidity, total darkness and 4°C). Sampling frequency varied depending on colony growth, but a minimum of 10 workers were sampled from every colony for every week of the experiment. To assess survival of workers, boxes were removed from the fridge every 48 h and were exposed to ambient light and temperature for 10 min. Workers were lightly prodded with forceps to verify whether they were alive and any that showed no sign of movement in 10 min were considered dead and stored at −20°C until further analyses. Live workers were placed back into the fridge. In order to identify potential confounding factors leading to mortality in the cold, other than colony age, we examined the correlation between survival length and worker age, body mass and oocyte size in a subset of the dead workers (Table S2).

Lipid analyses
To examine worker fat body lipid content, we compared lipid amounts in early-season (weeks 3–5) and late-season (weeks 8–11) workers, taken from four colonies. Workers were individually marked upon eclosion and worker age upon sampling was recorded (2–11 days). To control for size variation, worker body and eviscerated abdomen mass were measured prior to dissections. Lipid analysis was done as in Amsalem et al. (2015a) and Amsalem and Grozinger (2017). Briefly, the abdomen was separated from the thorax and eviscerated of all content except the fat body (i.e. the ovaries, gut and sting complex were removed), placed in 2% sodium sulfate and homogenized; 200 µl of the fat body homogenate was added to glass vials containing 2.8 ml chloroform–methanol mix. Vials were centrifuged (3000 g) and the supernatant was mixed with 2 ml of distilled H_2O, separating between the upper aqueous fraction and the lower organic fraction (lipids). The lower organic lipid fraction was separated and quantified using a vanillin–phosphoric acid reaction. A standard curve for lipids was created using five different concentrations of 0.1% vegetable oil diluted in chloroform. Absorbance values (OD at 525 nm) for each sample were measured with a Nanodrop One™ and converted to micrograms per worker, based on a formula calculated from the regression line derived from the calibration curve. Lipid quantity is presented as a percentage of abdomen mass to correct for body size.

Gene expression
Expression of genes was compared in 3-day-old workers sampled from early season colonies during weeks 2–4 (8 workers taken from three colonies) and from late-season colonies during weeks 8–10 (9 workers taken from three colonies; Table S1). Expression levels of *foxo*, *hsp70*, *hex1*, *menin*, *vg*, *mfe* and *pepck* were examined in the worker fat body. Target genes were selected based on their biological relevance to cold tolerance, lipid metabolism, stress and reproduction (Amsalem et al., 2015a; Hunt et al., 2007; Jedlička et al., 2016; Spacht et al., 2018). Design of the forward and reverse primers for each gene was done using primerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; see Table S3). Primers were designed to target the most conserved region between sequences.

Workers were individually marked in their parental colonies upon eclosion, flash-frozen 72 h later and stored at −80°C until use. To isolate the fat body tissue, workers were dissected using RNAlater to separate the abdomen from the thorax and remove all abdominal contents, including ovary tissue, sting complex and the gut. The remaining carcass and attached fat body were immediately placed in lysis buffer and stored at −80°C. Total RNA samples were extracted using the RNAeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized following the manufacturer's instructions using 200 ng of RNA (Applied Biosystems, Foster City, CA, USA).

Gene expression was measured on a QuantStudio 5 real-time PCR system (Applied Biosystems). For real-time PCR reactions, 2 µl of diluted cDNA was combined with 5 µl SYBR Green (Bioline, Luckenwalde, Germany), 0.2 µl of each forward and reverse primer and 4.6 µl DEPC-treated water. To control for PCR efficiency and individual differences across samples, we used two reference genes: *Arginine kinase* and *Phospholipase A2*. These genes have been previously used in several studies with *B. impatiens* (Amsalem et al., 2015a; Amsalem and Grozinger, 2017). Reactions were performed in triplicate for each of the samples and averaged for use in statistical analyses. Negative control samples and a water control were included for each plate. Expression levels of candidate genes were normalized to the geometric mean of the two housekeeping genes using the $2^{-\Delta Ct}$ technique (Vandesompele et al., 2002). For visual presentation, expression levels were normalized to the treatment with the lowest expression level.

Statistical analyses
All statistical analyses were conducted using JMP Pro software v.14.0.0 (SAS Institute Inc., Cary, NC, USA). Data were tested for normality using the goodness of fit test prior to statistical analysis and were analyzed using an ANOVA mixed model or a generalized linear model (GLM), accordingly. The effect of parental colony was examined and included in the model if found significant. Oocyte size data were analyzed using a GLM with colony age as the fixed effect, Poisson distribution, and log as the link function. Egg laying and worker size data were analyzed using an ANOVA mixed model with parental colony as a random effect followed by a *post hoc* analysis using Tukey's HSD Test. Worker mass was analyzed using a GLM with colony age as the fixed effect, a normal distribution, and log as the link function. Cold tolerance data were examined using Kaplan–Meier survival analyses. Lipid data were normally distributed following a log-transformation and analyzed using an ANOVA mixed model with parental colony as a random effect. Gene expression data were examined using either a *t*-test or a Wilcoxon ranked test, depending on the distribution of the data.

RESULTS

Effect of colony age on worker oocyte size and egg laying

Worker oocyte size (Fig. 1) was significantly affected by colony age (GLM, χ^2_{10}=85.88, P<0.001) but not by the parental colony (GLM, χ^2_1=1.64, P=0.20). The data clustered into three time points, separating between early season (<5 weeks), mid-season (6–9 weeks) and late season (>9 weeks). *Post hoc* contrast tests were performed to compare early-, mid- and late-season workers. Early-season workers (weeks 3–5) had a significantly smaller oocyte size compared with that of mid-season (6–9 weeks) workers (0.31 ±0.04 mm in early season versus 0.83±0.06 mm in mid-season, means±s.e.m.), in line with the pre-competition and competition phases of the colony (Duchateau and Velthuis, 1989). However, late-season workers (weeks 10–12) also showed a smaller oocyte size (0.36±0.03 mm) compared with that of mid-season workers, despite the inability of the queen to inhibit reproduction at this stage (Duchateau and Velthuis, 1988, 1989). The effect of colony age on worker oocyte size was the same regardless of whether worker body mass or worker size was included in the model.

As the presence of the queen may mask differences in worker reproduction early in the colony's life cycle, we also examined the effect of colony age on the physiological aptitude of workers to lay eggs in queenless conditions (Fig. 2). We placed pairs of newly eclosed workers in plastic cages for 10 days (n=422 workers) and compared their cumulative egg laying. Workers were collected from three time points during colony development, based on findings in the previous experiment (weeks 2–5, 6–8 and >9 weeks) and the overall longevity of the colonies used for these experiment (colonies were terminated at the age of 9–10 weeks, thus week 9 was considered late season). The average number of eggs was significantly affected by colony age ($F_{2,206}$=6.35, P=0.002). On average, workers that eclosed after week 9 laid significantly fewer eggs (16.77±1.5) compared with the number of eggs laid by workers that eclosed during weeks 2–5 (23.4±1.32) or weeks 6–8 (25.41± 1.42). The parental colony did not significantly affect the number of
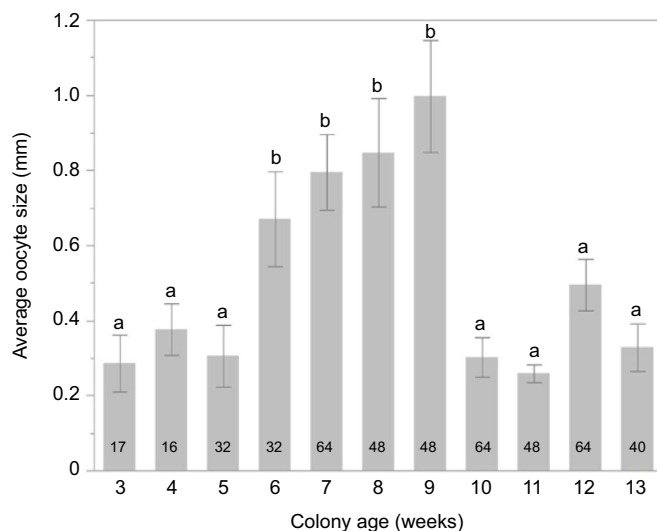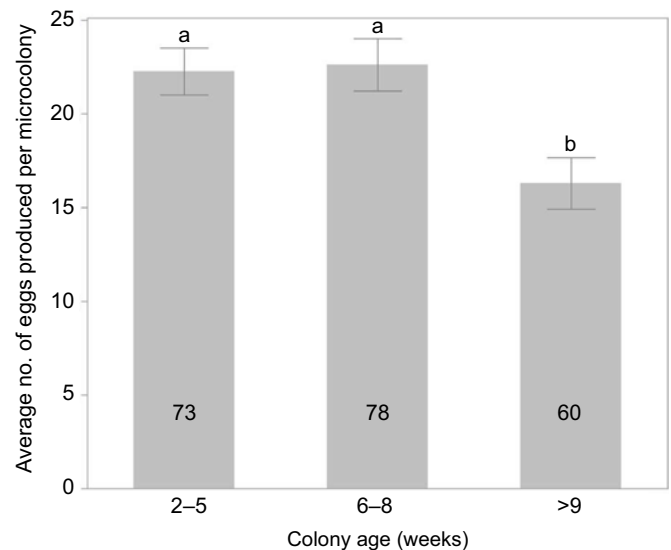
Fig. 2. Effect of colony age on the average cumulative number of eggs laid by queenless *B. impatiens* workers. Newly eclosed workers were sampled from four colonies during weeks 2–11 (calculated from the eclosion of the first worker) and kept in pairs for 10 days (n=211 cages). Numbers in bars represent the number of cages. Different lowercase letters indicate statistical differences at α=0.05. Data are presented as means±s.e.

eggs laid by workers ($F_{2,206}$=4.51, P=0.23), and thus was not included in the model.

Effect of colony age on worker cold survival

On average, workers survived 11 days under diapause-like conditions (3–5°C, 70% humidity) (Fig. 3). Colony age (in weeks) significantly affected the number of days workers survived in diapause-like conditions (Kaplan–Meier log-rank test, χ^2_{10}=81.5, P<0.001), with a difference of approximately 3 days between the average survival in the earliest week (week 3, 10.05±0.45 days) compared with the last week (week 13, 13.15±0.50 days). Significant differences were also found in the average survival of workers from wild and commercial colonies, with workers from wild-caught colonies surviving on average 11.59±0.2 days while workers from commercial colonies survived on average 10.8± 0.1 days (Kaplan–Meier log-rank test, χ^2_1=17.34, P<0.001). The parental colony had a significant effect on worker survival in both wild (Kaplan–Meier log-rank test, χ^2_1=9.85, P<0.001) and commercial colonies (Kaplan–Meier log-rank test, χ^2_3=133.57, P<0.001), with a difference of 3 days on average between the colonies that survived the longest and the shortest.

To test whether mortality in the cold could be attributed to factors other than colony age, we examined the correlation between the number of days survived in the cold and several potential confounding factors. Worker age was documented in two colonies where all workers were individually marked upon eclosion. We found a significant, though weak, negative correlation between worker age and cold survival in both colonies (colony 3: Pearson's r=−0.14, n=232, P=0.03; colony 4: Pearson's r=−0.21, n=743, P<0.001). The body mass of workers at death was examined in all six colonies but was weakly positively correlated with survival only in two colonies (colony 1: Pearson's r=0.13, n=322, P=0.02; colony 3: Pearson's r=0.22, n=233, P<0.001). Finally, no significant correlation was found between oocyte size at death and survival in any of the six colonies (Table S2).

Fig. 1. Effect of colony age on *Bombus impatiens* worker oocyte size. Workers were sampled from two colonies during weeks 3–13 (calculated from the eclosion of the first worker) and immediately frozen (n=472 workers). Numbers in bars represent the number of individuals. Different lowercase letters indicate statistical differences at α=0.05. Data are presented as means±s.e.m.
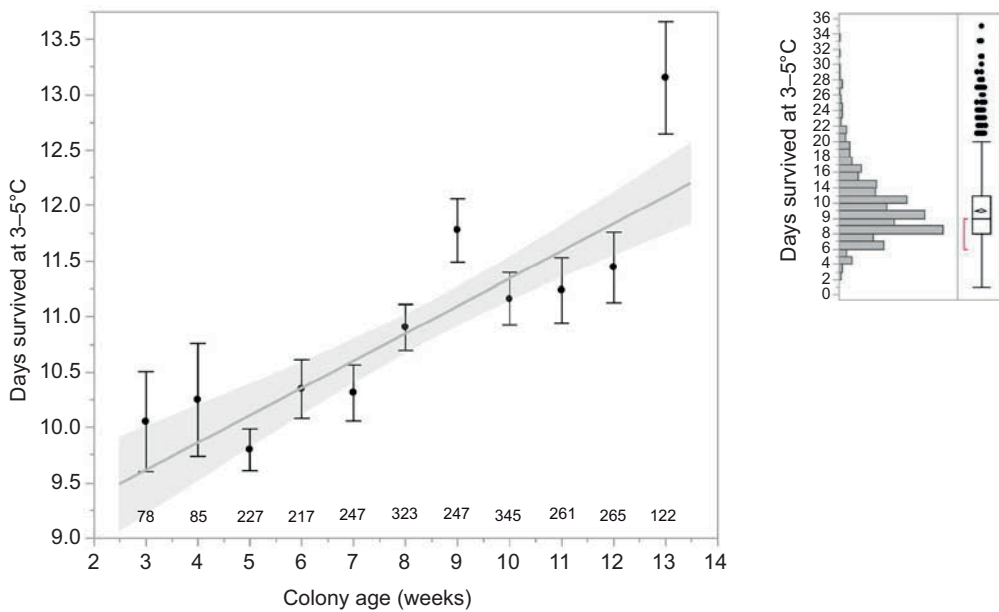
Fig. 3. Effect of colony age on *B. impatiens* worker survival in diapause-like conditions (3–5°C, 70% humidity). Groups of 10 workers were sampled every few days during weeks 3–13 (calculated from the eclosion of the first worker) from six colonies (four commercial and two wild-caught, n=2417 workers). The graph on the right represents the distribution of the number of days that workers survived in the cold out of the total sample size. The total number of workers sampled per week is shown under the data points. Statistical significance was accepted at α=0.05. Data are presented as means± s.e.m.

Effect of colony age on worker body mass and size

There was a significant effect of colony age on worker size ($F_{1,471}$=3.89, P=0.05) and worker body mass (GLM, χ^2_{10}=125.19, P<0.001; Fig. 4), although the size effect was slightly weaker than the mass effect. There was an increase in both size and mass over time, up to week 12, and a slight decrease in the last week (week 13). Colonies typically started showing signs of collapse after weeks 9–10 (see also Table S1 for the average survival in weeks of all colonies). Thus, we attributed the decrease in mass/size in week 13 to the final stage of the colonies and believe the consistent trend of increasing body size and mass in weeks 2–12 reflects the colony's normal development more accurately than the last week. Neither body mass (GLM, χ^2_1=2.76, P=0.10) nor size was affected by the parental colony ($F_{1,470}$=4.03, P=0.84).

Effect of colony age on worker fat body lipids

Lipid content in the fat body (normalized to eviscerated abdomen mass) was significantly higher in late-season compared with early-season workers (ANOVA mixed model: $F_{1,85.1}$=5.41, P=0.023; Fig. 5). Lipid content was not affected by the parental colony ($F_{1,85.1}$=5.41, P=0.33), and no significant correlation was found between worker age and fat body lipid content (Pearson's r=0.08, n=90, P=0.42).

Effect of colony age on worker gene expression

Expression levels of selected genes were examined in 3-day-old workers (Fig. 6), which are too young to activate their ovaries, ensuring that gene expression differences were not the result of a different reproductive state. Gene expression levels were compared using a *t*-test or Wilcoxon ranked test, depending on whether the
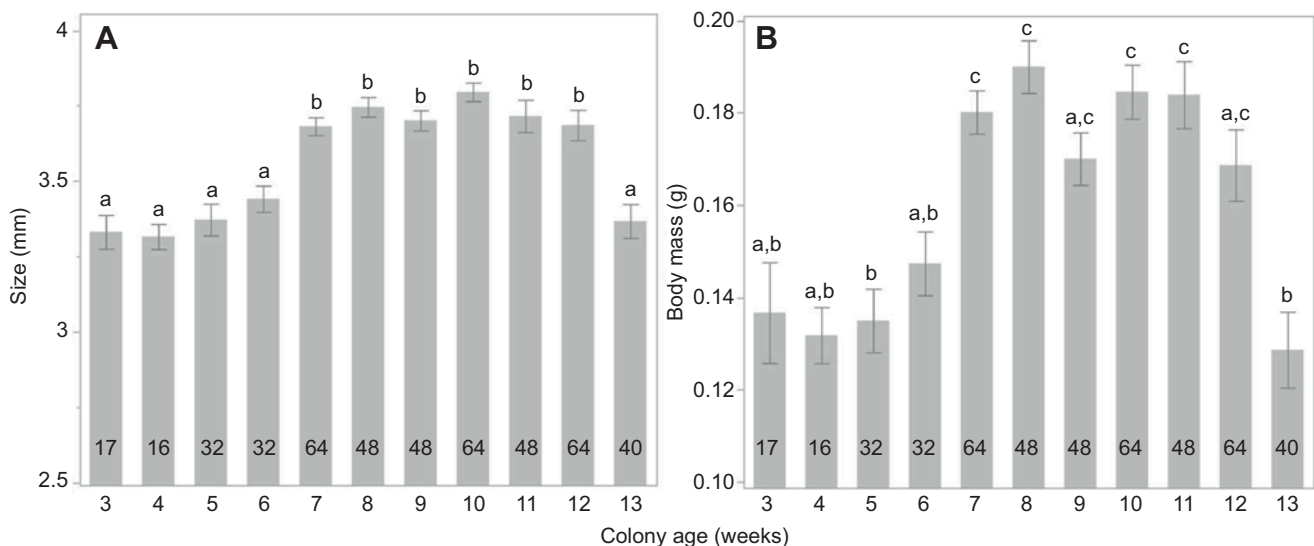

Fig. 4. Effect of colony age on *B. impatiens* worker size and mass. (A) Head width (as a measure of body size). (B) Body mass. Workers were randomly sampled from two colonies during weeks 3–13 (calculated from the eclosion of the first worker; n=472 workers). Numbers in bars represent the number of individuals. Different lowercase letters indicate statistical differences at α=0.05. Data are presented as means±s.e.m.
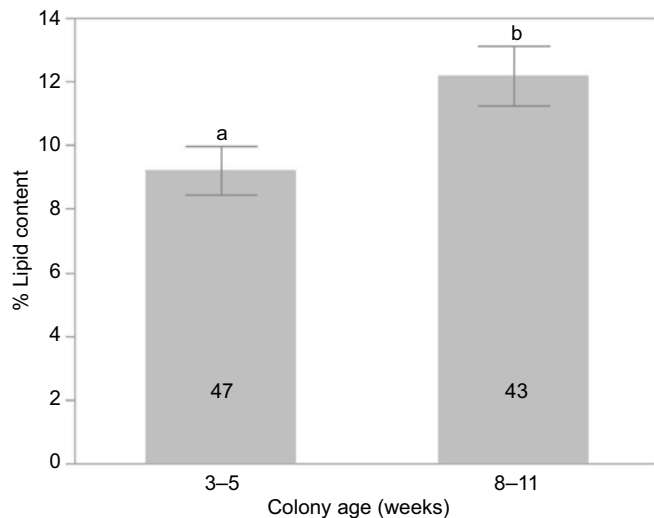
Fig. 5. Effect of colony age on the average percentage of fat body lipids in *B. impatiens* worker abdomens. Workers (*n*=90 workers) were sampled from four colonies during weeks 3–5 (early eclosing) versus weeks 8–11 (late eclosing) (where colony age was calculated from the eclosion of the first worker). Numbers in bars represent the number of individuals. Different lowercase letters indicate statistical significance at α=0.05. Data are presented as means±s.e.m.

data were distributed normally or not (see Materials and Methods). Expression levels of *menin*, *mfe* and *pepck* were significantly higher in early- compared with late-season same-age workers (*menin*: *t*-test: $t_{10.88}$=2.79, P=0.008; *mfe*: Wilcoxon ranked test: χ^2_1=5.36, P=0.02; *pepck*: *t*-test: $t_{12.16}$=2.25, P=0.02). Expression levels of *hex1* were significantly higher (Wilcoxon ranked test: χ^2_1=3.57, P=0.05) and expression levels of *hsp70* were marginally insignificantly higher (*t*-test: $t_{8.67}$=1.5, P=0.08) in late- versus early-season workers. The effect size found in our study, although not very high (typically 2–3 times higher in significantly upregulated genes), is equivalent to findings in previous studies. For example, in Amsalem et al., (2015a), where we compared the expression of *foxo* in diapausing versus non-diapausing *B. terrestris* queens, the expression levels were two times higher during diapause. *foxo* is a well-conserved gene known to be upregulated during diapause, so the size effect in this study for most of the genes is not out of norm. No significant differences were found in the expression levels of *vg* (χ^2_1=1.12, P=0.29) or *foxo* (χ^2_1=2.08, P=0.15).

DISCUSSION

Our study demonstrates that bumble bee workers exhibit physiological and gene expression pattern shifts from reproductive to a diapause-like profile as the colony ages, despite being reared under controlled temperature and humidity and provided with unlimited food. Overall, workers eclosing late in the colony cycle had less-activated ovaries under queenright conditions and laid fewer eggs under queenless conditions compared with workers eclosing early in the life cycle. On average, late-eclosing workers were larger, heavier, stored more lipids and were able to tolerate cold longer compared with early-eclosing workers. These observations are in line with an overall reproductive-like physiology early in the life cycle and a diapause-like physiology late in the life cycle.

These results are also summarized in Table 1. The main question to be answered is why maintaining and expressing these signatures is adaptive for bumble bee workers. Although not mutually exclusive, below we discuss four possible explanations for this phenomenon.

Physiological shift in workers is innate to workers: late-season worker physiology reflects an ancient diapause signature

Circannual patterns regulating developmental and physiological processes in individual insects, although uncommon, have been documented across numerous taxa (Miyazaki et al., 2014; Saunders, 2009). Perhaps the most well-known example is the carpet beetle (*Anthrenus verbasci*), whose larvae possess an endogenous annual rhythm that controls the onset of diapause (Blake, 1960; Nisimura and Numata, 2003). Dragonflies also use a circannual rhythm to alternate between phases of diapause and development over the course of a year (Norling, 1984; Saunders, 2010). In social insects, termites (*Coptotermes formosanus*) were shown to a have circannual rhythm regulating the developmental time of the soldier caste (Mao and Henderson, 2007). As circannual patterns allow an insect to synchronize their activities, physiology and behavior with annual environmental fluctuations, their adaptive value may be particularly important in seasonal environments where insects must survive unfavorable periods.

In species that inhabit seasonal environments, larger females are better suited to survive the winter diapause, a physiologically demanding period during their life cycle, and found a colony the following year. Selection based on body size can lead to the divergence of female castes and to fixed morphological differences between longer-lived, larger, diapause-capable queens and shorter-lived, smaller, helper workers that are unable to survive the winter.
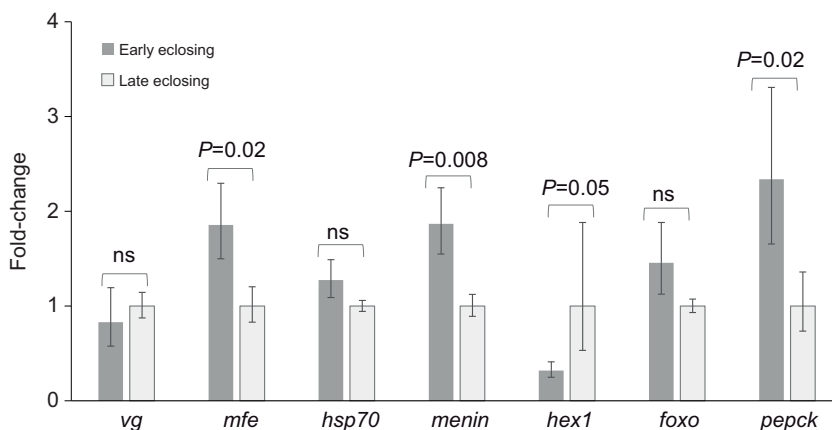

Fig. 6. Effect of colony age on expression levels of candidate genes in 3-day-old *B. impatiens* workers. Workers were sampled from six colonies during weeks 2–4 (early eclosing) and 8–10 (late eclosing) (*n*=17 workers). *P*-values denoting statistical significance (α=0.05) are given above bars. Data are presented as means±s.e.m.

Our finding that late-eclosing workers exhibit a more diapause-like profile compared with early-eclosing workers is similar to solitary multivoltine bee species (e.g. several *Xylocopa* species) whereby the early-season generation enter the reproductive cycle immediately following eclosion, and the late-season generation refrain from reproduction and enter diapause. This may suggest that the shift from immediate reproduction ('reproduce now') to diapause ('reproduce later') played an important role in the divergence of female castes in bumble bees, an idea that was formulated as the DGPH (Hunt, 2006).

The DGPH proposes that caste differences are driven by nutritionally sensitive ancestral genetic and physiological signatures that bifurcate during larval development in seasonal environments, resulting in two trajectories. (1) The 'G1 pathway', where early-season, poorly fed larvae develop slowly into adults with low fat and protein stores, and exhibit a reproductive-like physiology. These adults are predicted to seek protein upon eclosion and attempt to reproduce right away ('reproduce now'). (2) The 'G2 pathway', where late-season, well-fed larvae develop faster into adults with high fat and protein stores, and exhibit a diapause-like physiology that allows them to postpone reproduction until the following year ('reproduce later'). These two trajectories are hypothesized to give rise to a solitary ancestor with a facultative physiological and genetic signature from which small females, incapable of entering diapause (workers) and large, diapause-capable females (queens) have evolved.

Empirical support for the DGPH in insects is scarce and mostly indirect. Gyne-destined larvae of *Polistes* wasps (potential future queens) carry a diapause signature, exhibiting a longer developmental period and higher levels of hexamerin storage proteins that are associated with preparation for diapause (Hunt et al., 2007). Studies in *B. terrestris* showed that while the physiological and molecular changes associated with diapause are well conserved across species (Amsalem et al., 2015a; Ragland and Keep, 2017), genetic pathways regulating diapause in social insects have been co-opted to regulate the divergence of castes, as evidenced by substantial overlap between genes related to caste determination and diapause in bumble bees, and a shift in the role of key genes regulating diapause in *B. terrestris* compared with Diptera species (Amsalem et al., 2015a; Ragland et al., 2010). Finally, a recent phylogeny demonstrated that the most recent common ancestor of all bees likely exhibited developmental diapause, and that an ancestral shift from developmental diapause to adult diapause was an important preadaptation to sociality in bees (Santos et al., 2019).

Bumble bee female castes are fixed and are determined during larval development, leading to queens that are 3 times larger than workers (Couvillon and Dornhaus, 2010). These queens, unlike workers, are capable of surviving winter diapause and consequently of initiating a colony in the next spring. Our study did not directly examine the differences between queens and workers, but may provide insights into the evolutionary history of bumble bee castes by examining the shift in the seasonal signature that workers expressed. Bumble bee workers show large variability in body size (Couvillon and Dornhaus, 2010), which increases toward the end of the season (Knee and Medler, 1965; Shpigler et al., 2013), and also exhibit a shift from a reproductive to a diapause-like profile as the colony ages. These correlations may suggest that co-option of pathways regulating diapause to generate reproductive and non-reproductive phenotypes may have facilitated the evolution of the worker caste in species that evolved in seasonal environments. In support of that, late-eclosing workers in our study were larger and heavier (Fig. 4), and also had higher amounts of lipids in their fat

body (Fig. 5), both of which are characteristic of the diapause phenotype. They also showed higher cold tolerance (Fig. 3) and lower reproductive potential (Figs 1 and 2). Furthermore, genes that are predicted to be altered in preparation for diapause were differentially regulated in early- versus late-eclosing workers, in line with the DGPH (Fig. 6).

Environmental factors affecting caste divergence have been demonstrated in several species. For example, the production of discrete phenotypes in ants of temperate climates is influenced by the interaction between genetics and temperature (Libbrecht et al., 2013; Schwander and Keller, 2008), and the effect of nutritional cues during development on caste determination was demonstrated in bees, wasps, ants and termites (Corona et al., 2016). These examples and the data presented in this study suggest that the DGPH warrants further direct investigation. Future studies examining how seasonality affects larval development and body size across social species and manipulation of nutritional cues in larvae could further elucidate whether the diapause ground plan was a prerequisite for the development of reproductive and non-reproductive castes. A large-scale examination of genes that vary with seasonality in comparison with genes regulating diapause across species will further elucidate how conserved these pathways are and the role diapause may have played in the evolution of sociality and castes.

Physiological shift in workers is not innate: worker physiology is influenced by the social environment

The observed shift in worker physiology could also be a result of the social environment during larval development (i.e. the number of workers, the amount of food fed to the larvae and the presence of older nestmates). However, we reject this explanation for multiple reasons. In several of our experiments (Figs 1–5) we did not sample workers before weeks 2–3 (i.e. until a sufficient number of workers had accumulated) and controlled for the number of workers in the colony at the time of sampling, maintaining no more than 20–30 workers and the queen throughout the colony cycle. Therefore, the larger and heavier workers produced by the end of the life cycle were not taken care of by a higher number of individuals during their larval development. It should be noted, however, that a previous study found that *B. terrestris* worker body size was influenced by the social environment, with smaller-sized workers produced when reared by one versus 10 queenless workers (Shpigler et al., 2013). However, in the same study, no such difference was found under queenright conditions that better reflect our experimental design: workers that were reared by the queen and >10 workers were not larger than workers reared by a single worker in two out of three replicates (Shpigler et al., 2013). Interestingly, the sheer number of workers in late-stage colonies was proposed as a possible explanation for the decrease in queen dominance and the initiation of worker reproduction (Van Doorn and Heringa, 1986). However, this was not supported by our data showing that the shift in worker ovarian activation occurred despite the controlled colony size.

A previous study conducted in *B. terrestris* showed a pattern of worker ovary activation throughout colony development similar to the one we found (Fig. 1), with lower ovary activation in workers both very early and very late in the colony cycle (Bloch and Hefetz, 1999). The authors suggested that early inhibition of worker reproduction is induced by the queen while the later inhibition is caused by nestmate workers. This explanation, too, does not line up with our data as the frequent sampling of workers likely prevented the formation of such a hierarchy among workers.

Lastly, in all experiments, we provided the bees with unlimited food of the same quality, eliminating the possibility that fluctuations

in resource abundance or quality can explain the differences we observed. All colonies and individuals were kept in darkness, 60% humidity and 28–30°C, and overall, despite the control of worker demography, external conditions and queen presence, we still found clear differences in the physiology of early- and late-eclosing workers. However, our data are correlative in nature as they were collected from normally developing colonies. Thus, we cannot preclude the potential effect of other internal factors (e.g. brood number, brood composition, queen age, etc.) that were not controlled for.

Physiological shift in workers is innate to the colony: workers evolved to use seasonal cues as social cues determining female reproduction

A bumble bee colony typically contains hundreds of workers that emerge progressively over a period of approximately 3 months, followed by dispersal of reproductives and dissolution of the colony (Michener, 1974). In nature, this period is characterized by progressive decreases in floral resource availability (pollen and nectar), ambient temperature and photoperiod. During the first phase of the colony cycle, the queen exerts full control over worker reproduction but later in the season workers start activating their ovaries and laying eggs (Amsalem et al., 2015a; Duchateau and Velthuis, 1988). Workers are unmated, and thus can only produce haploid eggs that develop into males, and depend on the queen to produce the worker force to support the rearing of sexuals and to switch into rearing gynes, two events that are controlled solely by the queen (Cnaani et al., 2000; Duchateau and Velthuis, 1988). In *B. terrestris*, it was demonstrated that workers 'eavesdrop' on the queen by possibly monitoring her age and reproductive output and waiting for her to lay eggs that develop into gynes before attempting to lay eggs themselves (Alaux et al., 2006). Workers further closely monitor the queen's fecundity and initiate aggressiveness towards her when her productivity decreases (Almond et al., 2019), often resulting in matricide (Bourke, 1994). Therefore, it is beneficial for workers to be physiologically prepared to lay eggs in the event that the queen dies prematurely.

Seasonal limitations dictate that if workers wait too long to reproduce, even if they are successful in laying eggs, their eggs are unlikely to complete development as a result of decreased floral resources and temperatures. Furthermore, even in the case that eggs are fully developed to adults, the eclosion of males that were laid too late in the season is unlikely to synchronize with the eclosion of gynes, resulting in inability to find a mate. Therefore, while investing in reproduction early in the season is beneficial, investing in reproduction late in the season is likely to be futile.

In accordance with this explanation, we found that workers exhibit a higher reproductive capacity and output early in the life cycle and a diapause-like profile later in the life cycle. Likewise, the expression levels of *mfe*, a gene encoding the final enzyme in the biosynthesis of JH and associated with fertility (Jedlička et al., 2016), was downregulated in late- compared with early-eclosing workers. Additionally, *hex1*, a gene related to diapause preparation (Denlinger, 2002; Hunt et al., 2007; Zhou et al., 2006), was upregulated in workers eclosing later in the life cycle.

However, not all genes followed the expected trend. *vg*, for example, is a yolk-precursor protein whose expression is expected to increase in reproductives (thus, in early-eclosing workers). However, no significant differences were found in *vg* expression as the colonies aged. This lack of difference could be due to the association of *vg* with aggressive behavior in bumble bees rather than solely with reproduction (Amsalem et al., 2014). The lack of

differences in the expression levels of the other three genes (*foxo*, *hsp70* and *menin*) may be explained in the fact that mRNA expression does not always track protein levels, and some genes show higher individual variability in their expression than others (in a way that our sample size may have not captured). Diapause-related genes are also sometimes characterized with a complex expression pattern. *foxo*, for example, while downregulated in *B. terrestris* queens during diapause, is actually upregulated in queens prior to entering diapause and right after diapause (Amsalem et al., 2015a). Additionally, *menin*, a regulator of heat shock proteins and a general stress response (Zhao and Jones, 2012), was significantly downregulated in *B. terrestris* queens prior to entering diapause compared with queens already in diapause (Amsalem et al., 2015a).

With this in mind, we suggest that worker physiology throughout colony development matches the cascade of seasonal events occurring in the wild. Workers are expected to invest in their own reproduction when it is likely to be successful and invest in a diapause-like physiology (likely an attempt to survive the winter) when reproduction is unlikely to be successful. This explanation is fully supported by the physiological data but only partially supported by the gene expression results.

Physiological shift in workers is innate to the queen: queens lay eggs of different quality as they age

A final explanation we propose is that the observed shift in worker physiology results from the age of the queen. As social insect queens age, their reproductive output may shift to maximize their own fitness or as a result of stressors associated with aging. Bumble bee queens control their reproduction to delay production of gynes until later in the colony cycle, thereby reducing the time available for worker reproduction (Alaux et al., 2005). Studies examining the factors regulating gyne production have shown through cross-fostering experiments that the production of gynes can be accelerated by placing older queens (post-competition phase) in young colonies, suggesting that this event is controlled by the queen (Alaux et al., 2005, 2006). *Bombus terrestris* queens also decrease in fecundity following the switch point in the colony cycle (i.e. the transition of the queen to laying male eggs), reducing the frequency of egg-laying events as well as the number of eggs laid per cell (Almond et al., 2019). Queen age-induced shifts in offspring number and quality have also been identified in honey bees (*Apis mellifera caucasica*), where colonies containing 2 year old queens had 50% lower levels of brood production, reduced frames of adult bees and reduced brood quality compared with colonies headed by queens younger than 1 year old (Akyol et al., 2007). Furthermore, mortality of larvae increases with queen age, as does the size of the eggs produced (Al-Lawati and Bienefeld, 2009).

Overall, while these studies suggest that offspring physiology may shift as the queen ages, the function of producing diapause-like workers later in the season in bumble bees is not immediately clear as the shifts we observed in worker physiology over the colony's development do not necessarily indicate a reduction in the quality of the worker. In fact, with the exception of week 13, workers in older colonies had an increased size and mass and contained higher levels of fat body lipids, which could be interpreted as signs of increased worker quality. With that in mind, it should be noted that honey bee (*A. mellifera*) queens in smaller colonies or experiencing periods of nutritional stress were shown to produce larger eggs, likely as a way to increase the chance of their survival (Amiri et al., 2020). Although we did not control for the age of queen, our control of the environmental conditions and worker demography likely reduced the probability that the queen was simply responding to the colony

environment but cannot preclude the possibility that the physiological shift in workers is due to increased queen age.

Conclusions

Bumble bee workers were demonstrated to express a physiological signature corresponding to the age of the colony with a shift towards a diapause-like profile in late-eclosing workers. These findings may indicate that worker physiology is innate, but whether the signature is innate to workers, the colony or the queen warrants further study.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.218768.supplemental

References

Akyol, E., Yeninar, H., Karatepe, M., Karatepe, B. and Özkök, D. (2007). Effects of queen ages on Varroa (Varroa destructor) infestation level in honey bee (*Apis mellifera* caucasica) colonies and colony performance. *Ital. J. Anim. Sci.* **6**, 143-149. doi:10.4081/ijas.2007.143

Al-Lawati, H. and Bienefeld, K. (2009). Maternal age effects on embryo mortality and juvenile development of offspring in the honey bee (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am.* **102**, 881-888. doi:10.1603/008.102.0514

Alaux, C., Jaisson, P. and Hefetz, A. (2005). Reproductive decision-making in semelparous colonies of the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* **59**, 270-277. doi:10.1007/s00265-005-0035-6

Alaux, C., Jaisson, P. and Hefetz, A. (2006). Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal. *Behav. Ecol. Sociobiol.* **60**, 439-446. doi:10.1007/s00265-006-0184-2

Almond, E. J., Huggins, T. J., Crowther, L. P., Parker, J. D. and Bourke, A. F. G. (2019). Queen longevity and fecundity affect conflict with workers over resource inheritance in a social insect. *Am. Nat.* **193**, 256-266. doi:10.1086/701299

Amiri, E., Le, K., Melendez, C. V., Strand, M. K., Tarpy, D. R. and Rueppell, O. (2020). Egg-size plasticity in *Apis mellifera*: honey bee queens alter egg size in response to both genetic and environmental factors. *J. Evol. Biol.* doi:10.1111/jeb. 13589

Amsalem, E. and Grozinger, C. M. (2017). Evaluating the molecular, physiological and behavioral impacts of CO2 narcosis in bumble bees (*Bombus impatiens*). *J. Insect Physiol.* **101**, 57-65. doi:10.1016/j.jinsphys.2017.06.014

Amsalem, E. and Hefetz, A. (2010). The appeasement effect of sterility signaling in dominance contests among *Bombus terrestris* workers. *Behav. Ecol. Sociobiol.* **64**, 1685-1694. doi:10.1007/s00265-010-0982-4

Amsalem, E. and Hefetz, A. (2011). The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris*. *PLoS ONE* **6**, e18238. doi:10.1371/journal.pone.0018238

Amsalem, E., Galbraith, D. A., Cnaani, J., Teal, P. E. A. and Grozinger, C. M. (2015a). Conservation and modification of genetic and physiological toolkits underpinning diapause in bumble bee queens. *Mol. Ecol.* **24**, 5596-5615. doi:10. 1111/mec.13410

Amsalem, E., Grozinger, C. M., Padilla, M. and Hefetz, A. (2015b). The physiological and genomic bases of bumble bee social behaviour. In *Genomics, Physiology and Behaviour of Social Insects*, Vol. 48 (ed. Z. Amro and F. K. Clement), pp. 37-93. Adv In Insect Phys: Academic Press.

Amsalem, E., Malka, O., Grozinger, C. and Hefetz, A. (2014). Exploring the role of juvenile hormone and vitellogenin in reproduction and social behavior in bumble bees. *BMC Evol. Biol.* **14**, 45. doi:10.1186/1471-2148-14-45

Benton, T. and Bowler, D. (2012). Dispersal in invertebrates: influences on individual decisions. In *Dispersal Ecology and Evolution* (ed. J. Clobert, M. Baguette, T. G. Benton and J. M. Bullock), pp. 41-49: Oxford Scholarship.

Blacher, P., Huggins, T. J. and Bourke, A. F. G. (2017). Evolution of ageing, costs of reproduction and the fecundity–longevity trade-off in eusocial insects. *Proc. R. Soc. B Biol. Sci.* **284**, 20170380. doi:10.1098/rspb.2017.0380

Blake, G. M. (1960). Decreasing photoperiod inhibiting metamorphosis in an insect. *Nature* **188**, 168-169. doi:10.1038/188168a0

Bloch, G. (1999). Regulation of queen-worker conflict in bumble-bee (*Bombus terrestris*) colonies. *Proc. R. Soc. Lond. B* **266**, 2465-2469. doi:10.1098/rspb. 1999.0947

Bloch, G. and Hefetz, A. (1999). Regulation of reproduction by dominant workers in bumblebee (*Bombus terrestris*) queenright colonies. *Behav. Ecol. Sociobiol.* **45**, 125-135. doi:10.1007/s002650050546

Bourke, A. F. G. (1994). Worker matricide in social bees and wasps. *J. Theor. Biol.* **167**, 283-292. doi:10.1006/jtbi.1994.1070

Cameron, S. A., Hines, H. M. and Williams, P. H. (2007). A comprehensive phylogeny of the bumble bees (Bombus). *Biol. J. Linn. Soc.* **91**, 161-188. doi:10. 1111/j.1095-8312.2007.00784.x

Chapman, J. W., Reynolds, D. R. and Wilson, K. (2015). Long-range seasonal migration in insects: mechanisms, evolutionary drivers and ecological consequences. *Ecol. Lett.* **18**, 287-302. doi:10.1111/ele.12407

Cnaani, J., Robinson, G. E. and Hefetz, A. (2000). The critical period for caste determination in *Bombus terrestris* and its juvenile hormone correlates. *J. Comp. Physiol. A* **186**, 1089-1094. doi:10.1007/s003590000163

Cook, S. C., Eubanks, M. D., Gold, R. E. and Behmer, S. T. (2016). Summer and fall ants have different physiological responses to food macronutrient content. *J. Insect Physiol.* **87**, 35-44. doi:10.1016/j.jinsphys.2016.02.001

Corona, M., Libbrecht, R. and Wheeler, D. E. (2016). Molecular mechanisms of phenotypic plasticity in social insects. *Curr. Opin. Insect. Sci.* **13**, 55-60. doi:10. 1016/j.cois.2015.12.003

Couvillon, M. J. and Dornhaus, A. (2010). Small worker bumble bees (*Bombus impatiens*) are hardier against starvation than their larger sisters. *Insectes Soc.* **57**, 193-197. doi:10.1007/s00040-010-0064-7

Couvillon, M. J., Jandt, J. M., Duong, N. and Dornhaus, A. (2010). Ontogeny of worker body size distribution in bumble bee (*Bombus impatiens*) colonies. *Ecol. Entomol.* **35**, 424-435. doi:10.1111/j.1365-2311.2010.01198.x

Daimon, T. and Shinoda, T. (2013). Function, diversity, and application of insect juvenile hormone epoxidases (CYP15). *Biotechnol. Appl. Biochem.* **60**, 82-91. doi:10.1002/bab.1058

Danforth, B. N., Cardinal, S., Praz, C., Almeida, E. A. B. and Michez, D. (2013). The impact of molecular data on our understanding of bee phylogeny and evolution. *Annu. Rev. Entomol.* **58**, 57-78. doi:10.1146/annurev-ento-120811- 153633

DeGrandi-Hoffman, G., Gage, S. L., Corby-Harris, V., Carroll, M., Chambers, M., Graham, H., Watkins deJong, E., Hidalgo, G., Calle, S., Azzouz-Olden, F. et al. (2018). Connecting the nutrient composition of seasonal pollens with changing nutritional needs of honey bee (*Apis mellifera* L.) colonies. *J. Insect Physiol.* **109**, 114-124. doi:10.1016/j.jinsphys.2018.07.002

Denlinger, D. L. (2002). Regulation of diapause. *Annu. Rev. Entomol.* **47**, 93-122. doi:10.1146/annurev.ento.47.091201.145137

Duchateau, M. J. and Velthuis, H. H. W. (1988). Development and reproductive strategies in *Bombus terrestris* colonies. *Behavior* **107**, 186-207. doi:10.1163/ 156853988X00340

Duchateau, M. J. and Velthuis, H. H. W. (1989). Ovarian development and egg-laying in workers of *Bombus terrestris*. *Entomol. Exp. Appl.* **51**, 199-213. doi:10. 1111/j.1570-7458.1989.tb01231.x

Goehring, L. and Oberhauser, K. S. (2002). Effects of photoperiod, temperature, and host plant age on induction of reproductive diapause and development time in Danaus plexippus. *Ecol. Entomol.* **27**, 674-685. doi:10.1046/j.1365-2311.2002. 00454.x

Gotthard, K. (2001). Growth strategies of ectothermic animals in temperate environments. In *Environment and Animal Development* (ed. D. Atkinson and M. Thorndyke), pp. 287-304: Oxford BIOS Scientific.

Hines, H. M. (2008). Historical biogeography, divergence times, and diversification patterns of bumble bees (Hymenoptera: Apidae: Bombus). *Syst. Biol.* **57**, 58-75. doi:10.1080/10635150801898912

Hoover, S. E. R., Higo, H. A. and Winston, M. L. (2006). Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition. *J. Comp. Physiol. B* **176**, 55-63. doi:10.1007/s00360-005-0032-0

Hunt, J. H. (2006). Evolution of castes in Polistes. *Ann. Zool. Fenn.* **43**, 407-422.

Hunt, J. H., Kensinger, B. J., Kossuth, J. A., Henshaw, M. T., Norberg, K., Wolschin, F. and Amdam, G. V. (2007). A diapause pathway underlies the gyne phenotype in Polistes wasps, revealing an evolutionary route to caste-containing insect societies. *Proc. Natl. Acad. Sci. USA* **104**, 14020-14025. doi:10.1073/pnas. 0705660104

Jedlička, P., Ernst, U. R., Votavová, A., Hanus, R. and Valterová, I. (2016). Gene expression dynamics in major endocrine regulatory pathways along the transition from solitary to social life in a bumblebee, *Bombus terrestris*. *Front. Physiol.* **7**, 574. doi:10.3389/fphys.2016.00574

Karsai, I. and Hunt, J. H. (2002). Food quantity affects traits of offspring in the paper wasp *Polistes metricus* (Hymenoptera: Vespidae). *Environ. Entomol.* **31**, 99-106. doi:10.1603/0046-225X-31.1.99

Kipyatkov, V. E. (1993). Annual cycles of development in ants: diversity, evolution, regulation. Proceedings of the Colloquia on Social Insects 2, 25-48.

Knee, W. J. and Medler, J. T. (1965). The seasonal size increase of bumblebee workers (Hymenoptera: Bombus). *Can. Entomol.* **97**, 1149-1155. doi:10.4039/Ent971149-11

Koštál, V. (2006). Eco-physiological phases of insect diapause. *J. Insect Physiol.* **52**, 113-127. doi:10.1016/j.jinsphys.2005.09.008

Koštál, V. and Denlinger, D. L. (2011). Dormancy and developmental arrest in invertebrates Introduction. *J. Insect Physiol.* **57**, 537-537. doi:10.1016/j.jinsphys.2011.04.001

Libbrecht, R., Corona, M., Wende, F., Azevedo, D. O., Serrao, J. E. and Keller, L. (2013). Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. *Proc. Natl. Acad. Sci. USA* **110**, 11050-11055. doi:10.1073/pnas.1221781110

Mao, L. and Henderson, G. (2007). A case for a free-running circannual rhythm in soldier developmental time of Formosan subterranean termites. *Ins* **54**, 388-392. doi:10.1007/s00040-007-0956-3

Michener, C. D. (1974). *The Social Behavior of the Bees*. Cambridge, Massachusetts: Harvard University Press.

Mikkola, K. (2003). Red admirals Vanessa atalanta (Lepidoptera: Nymphalidae) select northern winds on southward migration. *Entomol. Fenn.* **14**, 15-24. doi:10.33338/ef.84168

Miyazaki, Y., Nisimura, T. and Numata, H. (2014). Circannual rhythms in insects. In *Annual, Lunar, and Tidal Clocks: Patterns and Mechanisms of Nature's Enigmatic Rhythms* (ed. H. Numata and B. Helm), pp. 333-350. Tokyo: Springer Japan.

Moret, Y. and Schmid-Hempel, P. (2009). Immune responses of bumblebee workers as a function of individual and colony age: senescence versus plastic adjustment of the immune function. *Oikos* **118**, 371-378. doi:10.1111/j.1600-0706.2008.17187.x

Morita, A., Soga, K., Hoson, T., Kamisaka, S. and Numata, H. (1999). Changes in mechanical properties of the cuticle and lipid accumulation in relation to adult diapause in the bean bug, *Riptortus clavatus*. *J. Insect Physiol.* **45**, 241-247. doi:10.1016/S0022-1910(98)00119-X

Nisimura, T. and Numata, H. (2003). Circannual control of the life cycle in the varied carpet beetle *Anthrenus verbasci*. *Funct. Ecol.* **17**, 489-495. doi:10.1046/j.1365-2435.2003.00753.x

Norling, U. (1984). Life history patterns in the northern expansion of dragonflies. *Adv. Odonatol.* **2**, 127-156.

Prokop, J., Dehon, M., Michez, D. and Engel, M. S. (2017). An early miocene bumble bee from Northern Bohemia (Hymenoptera, Apidae). *Zookeys* **710**, 43-63. doi:10.3897/zookeys.710.14714

Ragland, G. J. and Keep, E. (2017). Comparative transcriptomics support evolutionary convergence of diapause responses across Insecta. *Physiol. Entomol.* **42**, 246-256. doi:10.1111/phen.12193

Ragland, G. J., Denlinger, D. L. and Hahn, D. A. (2010). Mechanisms of suspended animation are revealed by transcript profiling of diapause in the flesh fly. *Proc. Natl. Acad. Sci. USA* **107**, 14909-14914. doi:10.1073/pnas.1007075107

Ramenofsky, M. and Wingfield, J. C. (2007). Regulation of migration. *Bioscience* **57**, 135-143. doi:10.1641/B570208

Rinehart, J. P., Li, A., Yocum, G. D., Robich, R. M., Hayward, S. A. L. and Denlinger, D. L. (2007). Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proc. Natl. Acad. Sci. USA* **104**, 11130-11137. doi:10.1073/pnas.0703538104

Santos, P. K. F., Arias, M. C. and Kapheim, K. M. (2019). Loss of developmental diapause as prerequisite for social evolution in bees. *Biol. Lett.* **15**, 20190398. doi:10.1098/rsbl.2019.0398

Saunders, D. S. (2009). Circadian rhythms and the evolution of photoperiodic timing in insects. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **34**, 301-308. doi:10.1111/j.1365-3032.2009.00699.x

Saunders, D. (2010). Controversial aspects of photoperiodism in insects and mites. *J. Insect. Phyiol.* **56**, 1491-1502. doi:10.1016/j.jinsphys.2010.05.002

Schwander, T. and Keller, L. (2008). Genetic compatibility affects queen and worker caste determination. *Science* **322**, 552. doi:10.1126/science.1162590

Shpigler, H., Tamarkin, M., Gruber, Y., Poleg, M., Siegel, A. J. and Bloch, G. (2013). Social influences on body size and developmental time in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* **67**, 1601-1612. doi:10.1007/s00265-013-1571-0

Sim, C. and Denlinger, D. L. (2008). Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. *Proc. Natl. Acad. Sci. USA* **105**, 6777-6781. doi:10.1073/pnas.0802067105

Spacht, D. E., Teets, N. M. and Denlinger, D. L. (2018). Two isoforms of Pepck in *Sarcophaga bullata* and their distinct expression profiles through development, diapause, and in response to stresses of cold and starvation. *J. Insect Physiol.* **111**, 41-46. doi:10.1016/j.jinsphys.2018.10.008

Starkey, J., Brown, A. and Amsalem, E. (2019a). The road to sociality: brood regulation of worker reproduction in the simple eusocial bee *Bombus impatiens*. *Anim. Behav.* **154**, 57-65. doi:10.1016/j.anbehav.2019.06.004

Starkey, J., Derstine, N. and Amsalem, E. (2019b). Do bumble bees produce brood pheromones? *J. Chem. Ecol.* **45**, 725-734. doi:10.1007/s10886-019-01101-4

Tufail, M., Nagaba, Y., Elgendy, A. M. and Takeda, M. (2014). Regulation of vitellogenin genes in insects. *Entomol. Sci.* **17**, 269-282. doi:10.1111/ens.12086

Van Doorn, A. and Heringa, J. (1986). The ontogeny of a dominance hierarchy in colonies of the bumblebee *Bombus terrestris* (Hymenoptera, Apidae). *Insectes Soc.* **33**, 3-25. doi:10.1007/BF02224031

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034. doi:10.1186/gb-2002-3-7-research0034

Wheeler, D. E. (1986). Developmental and physiological determinants of caste in social hymenoptera: evolutionary implications. *Am. Nat.* **128**, 13-34. doi:10.1086/284536

Wikelski, M., Moskowitz, D., Adelman, J. S., Cochran, J., Wilcove, D. S. and May, M. L. (2006). Simple rules guide dragonfly migration. *Biol. Lett.* **2**, 325-329. doi:10.1098/rsbl.2006.0487

Yang, J., Kalhan, S. C. and Hanson, R. W. (2009). What is the metabolic role of phosphoenolpyruvate carboxykinase? *J. Biol. Chem.* **284**, 27025-27029. doi:10.1074/jbc.R109.040543

Zhao, L. and Jones, W. A. (2012). Expression of heat shock protein genes in insect stress responses. *Invert. Surviv. J.* **9**, 93-101.

Zhou, X., Oi, F. M. and Scharf, M. E. (2006). Social exploitation of hexamerin: RNAi reveals a major caste-regulatory factor in termites. *Proc. Natl. Acad. Sci. USA* **103**, 4499-4504. doi:10.1073/pnas.0508866103

Supplemental Information:

Table S1*:* Information on development and size of colonies used in this study. The number of workers sampled from different colonies for each of the different analyses is indicated in parentheses.

Colony	Sample size	Gynes Produced	Males Produced	# Workers Produced
1	Cold tolerance (435), ovaries, size and mass (231)	Never	Week 13	674
2	Cold tolerance (485), ovaries, size and mass (241)	Never	Week 13	759
3	Cold tolerance (233), lipids (26)	Week 6 (n=59)	Week 8	233
4	Cold tolerance (752), lipids (33)	Never	Week 7	752
5 (wild)	Cold tolerance (136), lipids (14)	Week 8 (n=30)	Week 12	230
6 (wild)	Cold tolerance (375), lipids (17)	Never	Week 13	444
7	Egg laying (118)	Week 10 (n=57)	Week 9	150
8	Egg laying (124)	Week 9 (n=75)	Week 9	650
9	Egg laying (144)	Week 10 (n=175)	Week 10	500
10	Egg laying (58), Gene expression (2)	Week 8 (n=94)	Unknown	Unknown
11	Gene expression (3)	Unknown	Unknown	Unknown
12	Gene expression (3)	Unknown	Unknown	Unknown
13	Gene expression (3)	Unknown	Unknown	Unknown
14	Gene expression (3)	Unknown	Unknown	Unknown
15	Gene expression (3)	Unknown	Unknown	Unknown

Table S2. The correlation between the numbers of days workers survived in 3-5°C and worker's age, body mass and oocyte size. In two of the colonies, workers were tagged and their age upon mortality was known. Body mass and ovaries were measured in workers after their death and tested against the individual survival data of workers.

Analysis	Individual Age	Body mass	Ovary activation
1	*Not examined*	r=0.13, n=322, p=0.02	r=0.05, n=278, p=0.32
2	*Not examined*	r=0.00, n=344, p=0.93	r=0.04, n=308, p=0.44
3	r= -0.14, n=232, p=0.03	r=0.22, n=233, p<0.001	r=0.03, n=28, p=0.85
4	r=-0.21, n=743, p<0.001	r=0.00, n=752, p=0.58	r=-0.02, n=43, p=0.86
5	*Not examined*	r=0.06, n=136, p=0.48	r=0.167, n=51, p=0.24
6	*Not examined*	r=0.04, n=376, p=0.43	r=0.07 n=58, p=0.62

Table S3. List of genes examined in this study, their accession numbers, and primer sequences

Gene	Accession Numbers	Forward primer	Reverse Primer
Arginine kinase (HKG)	XM_012391881.2	GTTGGTAGGGCAGAAGGTCA	AGGTCTACCGTCGTCTGGTG
Phospholipase A2 (HKG)	XM_003491149.3	CATTTCCGCAAGTGGTAGGT	GGTCACACCGAAACCAGATT
Forkhead transcription factor (*foxo*)	XM_024366034.1 XR_002946280.1 XR_001102115.2 XR_001102114.2 XM_012382794.2	CTCCCATCAATTGTCCCATC	CAACAACAATCGCAAACAGG
Hexamerin1 (*hex1*)	XM_012390757.2	GAATTGCCAAATCGAGAGGA	TCATTCAATTGCCACCCCCA
Heat shock protein 70 (*hsp70*)	XM_003491915.3 XM_012389299.2	TCATTGTCTGGTCGCAGGTC	ACCTATGGATGAAGAAAGGCGT
Menin (*menin*)	XM_003488543.3	AACCGATCACTGGCAACCAT	GATCACGCCTGGGTGGTTTA
Methyl farneosoate epoxidase (*mfe*)	XM_003484680.3	CAGCCGCCAATATGATACCT	GATCACGCCTGGGTGGTTTA
Phosphoenolpyruvate carboxykinase (*pepck*)	XM_012381009.2	GTTGGTAGGGCAGAAGGTCA	GCTCTACGCGACCATCTCTC
Vitellogenin (*vg*)	XM_003492229	CAGCCGCCAATATGATACCT	CCCTCCGTTCGAAGTGATAA