The role of insect diapause in the divergence of social female castes: Insights from primitively eusocial bumble bee workers

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Abstract

A key question in social evolution is how worker and queen castes diverged to give rise to social insect societies. The Diapause Ground Plan hypothesis (DGPH) proposes that caste differences are driven by ancestral genetic and physiological signatures that are triggered during larval development. This has been demonstrated in Polistes wasps, but the applicability of this theory to other species remained unexplored.

Primitively eusocial bumble bees are excellent models to examine the DGPH since they originated in seasonal environments, where the ability to survive diapause may have played an important role in the evolution of female castes. In bumble bees, differences between female castes are limited to body size, with larger females (queens) that undergo a winter-diapause and found a colony the next year, and smaller, short lived workers, that attempt to produce males. The bumble bee worker caste is an excellent system to examine a seasonal signature in females since they are all morphologically identical and are produced in various body sizes throughout the colony lifecycle.

Here, we compared the physiology and gene expression of early- and late-season *Bombus impatiens* workers across the colony lifecycle. We hypothesized that if seasonality has played an important role in the divergence of castes in Bombus, workers should maintain a seasonal signature, with early-season workers exhibiting a reproductive signature and late-season workers exhibiting a diapause signature. We found that late- but not early-season workers exhibit a diapause-like phenotype, characterized by lower reproductive potential, greater cold tolerance, increased size and fat-body lipid reserves and a diapause-like gene expression profile. These findings suggest that diapause may have played a critical role in the divergence of castes in social insects that evolved under seasonal conditions.

Introduction

Eusociality has evolved multiple times in insects (Danforth et al. 2013; Hughes et al. 2008) and reached its most advanced form in societies where reproduction is monopolized by a single or a few females (queen/s) while the remaining females (workers) function as helpers. Female castes in advanced eusocial species are often characterized with fixed morphological differences, however, such differences are not required within the strict definition of eusociality and are considered a derived trait of advanced eusocial societies (Beekman et al. 2006; Wilson 1971). Indeed, in many primitively eusocial species such as *Polistes* wasps (Hunt 2007; West-Eberhard 1969) and *Ponerine* queenless ants (Monnin and Peeters 1999) where a clear reproductive skew has evolved, all females are morphologically identical and have retained their ability to reproduce (Michener 1974). In more advanced eusocial insect species, there are irreversible morphological and physiological differences between castes (Wilson and Holldobler 2005). For example, *Apis mellifera* queens have lost the pollen-collecting structure possessed by the functionally sterile workers (Michener 1974), and workers of several species of stingless bees (Boleli et al. 2000) and ants (Khila and Abouheif 2008) exhibit developmental degeneration of their ovaries.

Primitively annual eusocial insects such as paper wasps, bumble bees, and sweat bees exhibit intermediate caste differences among females that are limited to body size (Alford 1975; Richards and Packer 1996; West-Eberhard 1969) and therefore provide excellent model systems to examine the evolutionary development of castes (Beekman et al. 2006). In species that inhabit seasonal environments, larger females are better suited to survive the winter-diapause, a physiologically demanding period during their lifecycle, 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, an idea that was formulated as the Diapause Ground Plan Hypothesis (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 exhibiting a reproductive-like

physiology. These adults are predicted to seek protein upon emergence 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 exhibiting 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. Gyne-destined larvae of *Polistes* wasps (potential future queens capable of entering a winter-diapause) exhibit a longer developmental period and higher levels of hexamerin storage proteins that are associated with preparation for diapause (Hunt et al. 2007). Study in *Bombus terrestris* showed that while the physiological and molecular changes associated with diapause are well-conserved across species (Amsalem et al. 2015; Ragland and Keep 2017), genetic pathways regulating diapause in social insects have been co-opted to regulate the divergence of castes, evidenced by substantial overlap between genes related to caste determination and diapause in bumble bees, and a shift in key genes regulating diapause in *Bombus terrestris* compared with Diptera species (Amsalem et al. 2015; 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 insects (Santos Priscila Karla et al. 2019). However, empirical studies directly examining the DGPH in species other than *Polistes* are lacking, thus the applicability of this theory to other social insect species that evolved in seasonal environment remains unexplored.

Bumble bees are an excellent model system to examine the DGPH since they originated in temperate climate regions where the ability to survive cold temperatures may have played an important role in their social evolution (Hines 2008; Prokop et al. 2017). All species within Bombini go through adult diapause and are hypothesized to evolve from an ancestor that went through developmental diapause (Santos Priscila Karla et al. 2019). Bumble bee colonies are founded by a single queen that produces hundreds of workers across the season, that are morphologically identical to the queen. While workers retain their ability to reproduce (as well as their mating organs and spermatheca), they are reproductively inhibited by the presence of the

queen and her brood during the first social phase of the colony (pre-competition phase) (Duchateau and Velthuis 1988; Starkey et al. 2019). Towards the end of the colony's lifecycle, gynes are produced by the queen and worker reproduction initiates in the so-called "competition phase" (Duchateau and Velthuis 1988). Bumble bee workers were described as "dwarf queens" (Cnaani and Hefetz 2001), and differ from gynes in the size of their body, with threefold differences between the smallest gyne and the largest worker (Goulson et al. 2002; Michener 1974). While gynes are only produced at the end of the season and size differences between workers and gynes are fixed, workers are produced continuously throughout the season and demonstrate even greater plasticity in their body size, up to tenfold differences in body mass among workers (Couvillon and Dornhaus 2010) making them an excellent model system to examine their physiology and genetics for season-dependent signatures.

In this study, we endeavored to examine the role of diapause in the evolution of sociality by comparing physiology and gene expression profiles of early-and late-emerging *Bombus impatiens* workers across the colony lifecycle. We hypothesized that if seasonality has played an important role in the divergence of castes in Bombus, workers should maintain a seasonal signature associated with the age of the colony, with early-season workers exhibiting a reproductive-like signature and late-season workers exhibiting a physiological and genetic diapause-like signature. To examine this, we used an integrative approach and compared worker reproduction, body size and mass, lipid content in the fat body, cold tolerance and expression of genes related to diapause and reproduction throughout the colony's development. In Table 1 we provide the list of variables examined in this study, the predictions stemming from Hunt's theory regarding each of the variables and compared them with the findings in this study.

Materials and Methods

Bumble bee rearing

Bombus impatiens colonies (n=13, Table S1) were obtained from BioBest at the age of 2-3 weeks after the emergence of the first worker or reared in the lab from gynes produced in BioBest colonies using a standard CO_2 protocol (Amsalem and Grozinger 2017). Two more colonies were reared from queens caught in the wild in spring of 2018 in Newport and

Landisville, Pennsylvania. All colonies were kept in darkness, 60% humidity and 28-30°C. Pollen and 60% sucrose solution were provided *ad-libitum.* Bumble bees produce sexuals using a 'bang bang' strategy: an abrupt switch from the exclusive production of workers to the exclusive production of sexuals (Duchateau and Velthuis 1988). In all experiments, workers were sampled throughout the natural development of the colonies. Workers emerging early and late in the colony lifecycle (measured from the emergence of the first worker) were considered early-and late-season workers, respectively. Sampling from the parental colonies began at approximately two weeks after the emergence of the first worker and continued until workers were no longer produced. Colonies were terminated when only males or gynes were exclusively produced for five consecutive days. Sampled occurred at a rate that ensured colony size was maintained between 20 and 30 workers. We collected information about colony life span, the production of first sexual (males and gynes) and the number of workers each colony produced (Table S1). 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=2,417), and fat body gene expression (n=17).

Ovary activation, body mass and size

To examine the hypothesis that late-season workers have lower reproductive potential but are larger and heavier compared with early-season workers (Table 1), groups of eight randomly sampled workers were removed from their parental queen-right colonies (i.e., queen is present) every 2-4 days throughout the colony's natural lifecycle (weeks 3-13) and kept at -20° C until analysis. 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 hypothesis that early-season workers have higher reproductive output compared with late-season workers (Table 1), we counted the number of eggs laid by same-age workers sampled from four colonies throughout the entire lifecycle (weeks 2-10). Two newly-emerged workers of approximately similar size were removed from their parental colony and placed into a cage (11 cm diameter x 7 cm tall) where they were provided *ad libitum* pollen and 60% sucrose syrup for 10 days. Cages were inspected for the total number of eggs laid by workers on day 10.

Cold tolerance

To examine the hypothesis that late-season workers have higher cold tolerance compared with early-season workers, we examined cold tolerance using a modified protocol from (Karsai and Hunt 2002) in groups of 10 workers that were removed from their colony throughout the entire colony lifecycle (weeks 3-13). Groups were placed in the refrigerator in a 28 x 22 x 5 cm cardboard boxes under conditions 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 hours and were exposed to ambient light and temperature for ten minutes. Workers were lightly prodded with forceps to verify whether they were alive and any that showed no sign of movement in 10 minutes 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 cold, other than the colony's age, body mass and oocyte size were examined in a subset of the dead workers. To test the effect of age on mortality in cold, all the workers in two colonies were tagged daily with an individual number and put back into the colony.

Lipid analyses

To examine the hypothesis that late-season workers have higher lipid content compared with early-season workers (Table 1), we compared lipid amounts in early-season (weeks 3-5) and late-season (weeks 8-11) workers, taken from four colonies. Workers were individually tagged upon emergence and worker age upon sampling was recorded (2-11 days). To control for size variation, worker body and abdomen mass were measured prior to dissections. Lipid analysis was done as in (Amsalem et al. 2015; 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), and placed in 2% sodium sulphate. Two hundred microliters of the fat body homogenate were added to glass vials containing 2.8 mL chloroform/methanol mix. Vials were centrifuged (x3000g) 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 upper organic phase (lipids) 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 525) 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. The amounts of lipids are presented as percentages of the abdomen mass to correct for body size.

Gene expression

To examine the hypothesis that late-season workers have a diapause profile whereas early-season workers have a reproductive profile, we compared gene expression of diapause and reproduction related genes in 3-day old early- (week 3) and late- (week 10) season workers (n=17) that were taken from six colonies. Expression levels of *foxo, hsp70, hex1, menin, vg, mfe,* and *pepck* were examined in the workers' fat body. Candidate genes were selected based on their biological relevance to diapause and reproduction (Amsalem et al. 2015; Hunt et al. 2007; Jedlicka et al. 2016; Spacht et al. 2018). Design of forward and reverse primers for each gene was done using the primerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are provided in Table S3. Primers were designed to target the most conserved region between sequences.

Newly emerged workers were tagged in their parental colonies upon emergence, flash-frozen 72 hours later and stored at -80°C until use. To isolate fat body tissue, workers were placed on dry ice and 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 manufacture instructions. cDNA was synthesized following the manufacturer's instructions using 200 ng of RNA (Applied Biosystems).

Gene expression was measured on a QuantStudio 5. For real time PCR reactions, 2 µL of diluted cDNA were combined with 5 µL SYBR Green (Bioline, Luckenwalde, Germany), 0.2 µL of each forward and reverse primer and 4.6 µL DEPC-water. To control for PCR efficiency and individual differences across samples, we used two housekeeping genes: *Arginine kinase* and *Phospholipase A2*. These genes have been previously used in several studies with *B. impatiens* (Amsalem et al. 2015; 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 present on each plate. Expression levels of candidate genes were normalized to the geometric mean of the two housekeeping genes using the $2^{-\Delta Ct}$ technique. 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 Goodness of fit test prior to the 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 generalized linear model (GLM) with colony's age as the fixed effect, Poisson distribution, and log-as-link function (over-dispersion was included). Egg laying and worker size data were analyzed using an ANOVA mixed model with parental colony as a random effect. Post-hoc analyses were performed with Tukey's HSD Test. Worker mass was analyzed using a GLM with colony's age as the fixed effect, a normal distribution, and log-as-link function (over-dispersion was included). 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

The effect of colony's age on worker oocyte size and egg laying

Worker ooocyte size (Figure 1) was significantly affected by the colony's 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). Post-hoc contrast tests showed that early-season workers (weeks 3-5) had significantly smaller oocyte size compared with mid-season (6-9 weeks) workers (0.31±0.04 mm in early vs. 0.83±0.06 mm in mid-season, means ± SEM), 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 smaller oocyte size (0.36±0.03 mm) compared with mid-season, despite the inability of the queen to inhibit reproduction at this stage (Duchateau and Velthuis 1988).

Since the presence of the queen may mask the differences in worker reproduction early in the colony's lifecycle, we repeated the experiment with queen-less workers sampled upon emergence (Figure 2). To test the effect of colony's age on the physiological aptitude of queen-less workers to lay eggs, we paired newly emerge workers at different colony ages (weeks 2-5, 6-8 and >9 weeks), according to the findings in the previous experiment, kept them in separate cages for 10 days (n=422 workers) and compared their cumulative egg laying. The average number of eggs was significantly affected by colony age ($f_{2,206=}$ 6.35, p=0.002). On average, workers that emerge after week nine of the colony laid significantly fewer eggs (16.77 ± 1.5) compared to those laid by workers that emerge 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 eggs laid by workers (p=0.23), and thus was not included in the model.

The effect of colony's age on workers cold survival

On average, workers survived 11 days under diapause-like conditions (3-5°C, 70% humidity) (Figure 3). Colony's 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 three days between the average survival in the earliest week (week 3, 10.05±0.45) compared with the latest week (week 13, 13.15±0.50). 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 three days on average between the colonies who survived the longest and the shortest.

To test whether mortality in cold could be attributed to factors other than colony's age, we examined the correlations between the number of days survived in cold and several potential confounding factors. Worker's age was known in two colonies where all workers were tagged upon emergence. We found a significant, though weak, negative correlation between age and cold survival in both colonies (Colony 3: Pearson's $r= -0.14$, $n=232$, $p=0.03$; Colony 4: Pearson's $= -0.21$, $n=743$, $p<0.001$). The body mass of workers upon 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 upon death and survival in any of the six colonies ($p>0.17$).

The effect of colony's age on worker body mass and size

There was a significant effect of colony's age on worker size ($f_{1,471}=3.89$, $p=0.05$) and worker body mass (GLM, $\chi^2_{10}=125.19$, $p<0.001$) (Figures 4a and 4b, respectively). There was an increase in both size and mass over time, up to week 12, and a slight decrease during the last week (week 13). Neither body mass or size were affected by the parental colony ($p>0.23$).

The effect of colony's age on worker fat body lipids

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

The effect of colony's age on worker gene expression

Expression levels of selected genes were examined in 3-day old workers (Figure 6). We chose 3-day old workers since they are too young to activate their ovaries, ensuring that gene expression differences are not the result of a different reproductive state. Genes were selected based on their role in diapause and reproduction. We examined (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 general stress response previously found to be upregulated during diapause in *Bombus terrestris* queens (Amsalem et al. 2015); (3) *hsp70:* a heat shock protein that is produced in response to cold in insects (Rinehart et al. 2007) and 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 Juvenile hormone (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*: the major egg yolk protein female insects invest in the ovaries (Tufail et al. 2014) that is predicted to be downregulated during diapause and upregulated in reproductives. Gene expression levels were compared using a T-test or Wilcoxon ranked test, depending on whether the data distributed normally or not (see 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 higher but insignificant (T-test: t (8.67)=1.5, p=0.08) in late- vs. early-season workers. No significant differences were found in the expression levels of *vg* or *foxo (*p>0.15).

Discussion

How female worker castes evolved is a key question in social evolution (Wheeler 1986; Wilson and Holldobler 2005). Insect groups showing a range of social organizations (*e.g.*, halictid bees) or primitively eusocial insects featuring the intermediate stages of caste differences (*e.g.*, Bombus and Polistes wasps), are excellent model systems to examine how eusociality, or more advanced forms of sociality, evolved (Rehan and Toth 2015).

Here we studied possible shifts in traits regarding the ability to enter diapause between early- and late-season workers and demonstrate that bumble bee workers show different physiological characteristics along colony development. Our finding that late-season workers exhibit traits favoring entering into diapause is similar to solitary multivoltine bee species (e.g., several

Xylocopa species) whereby the early-season generation enter the reproductive cycle immediately following emergence, and the late-season generation refrain from reproduction and enter into diapause. We propose that the shift from immediate reproduction ('reproduce now') to diapause ('reproduce later') played an important role in the transition from solitary to social living and the consequent evolution of castes in bees.

Overall, we show that despite colonies being reared under controlled temperature and humidity and provided unlimited food, workers exhibit physiological and genetic differences corresponding to a reproductive signature in early-season and a diapause signature in late-season. Workers emerging late in the season had less activated ovaries under queen-right conditions and laid fewer eggs under queen-less conditions compared with early-season workers. On average, late-season workers were larger, heavier, stored more lipids and were able to tolerate cold longer compared with early-season workers. While the physiology associated with seasonality fully matched predictions of the DGPH (Table 1), the gene expression data were mixed.

Genes that are predicted to be altered in preparation to diapause such as *hex1* (expression is expected to increase) and *pepck* (expression is expected to decrease) and genes related to juvenile hormone biosynthesis and reproduction such as *mfe* (expression is expected to decrease) were differentially regulated in early vs. late-season workers, in line with the DGPH. However, 3 of the genes we tested did not significantly differ between early and late-season workers (*foxo*, *hsp70* and *vg*) and one gene (*menin*) was significantly regulated in the opposite direction. However, given that RNA expression do not always track the protein levels, and that some genes show higher individual variability in their expression than others (in a way that our sample size may have not captured), we think these results coupled with the extensive physiological data provide substantial support for the DGPH. Furthermore, although correlative in nature, these physiological and genetic differences are particularly striking in a controlled environment and in same-age workers suggesting that, in line with the DGPH, workers may still maintain and express an ancient seasonal signature regardless of the environmental conditions they are in.

Among the genes that were significantly regulated are a few of a particular interest. *Hex1* encodes to storage proteins that accumulate in individuals destined for diapause (Denlinger 2002) as was shown in *Polistes* (Hunt et al. 2007), and also suppresses juvenile hormone production (Zhou et al. 2006). *Hex1* was upregulated in late-season workers, in line with a

diapause signature. The expression levels of *pepck*, a gene coding to a metabolic enzyme used during gluconeogenesis (Spacht et al. 2018), were down-regulated in late-season workers, likely indicating reduced gluconeogenesis and a conservation of energy stores in preparation to diapause. *Methyl farnesoate epoxidase, (mfe*), a gene associated with fertility, was upregulated in early-season workers. *Mfe* encodes to the final enzyme in the biosynthesis of JH, the main gonadotropin in insects, and is often examined as a proxy for reproduction (Jedlicka et al. 2016). *Mfe* was upregulated in reproductive queens and fertile workers in other species of *Bombus* (Jedlicka et al. 2016). To compare gene expression, we used 3-day old workers. At this age, workers have inactivated ovaries, yet early-season workers at this age showed higher *mfe* expression levels compared with same-age late-season workers, likely indicating of a greater reproductive potential of workers emerging early in the season.

We found no differences in the expression levels of *vg*, the main egg-yolk protein females invest in their ovaries (Tufail et al. 2014). However, previous studies suggested *vg* expression in bumble bees is only secondary associated with reproduction and primarily associated with aggressive behavior in workers (Duchateau and Velthuis 1988). No significant differences were found also in *foxo* and *hsp70,* and *menin* was significantly differently regulated in the opposite direction. We predicted *hsp70* and *menin* to be higher and *foxo* to be lower during diapause, however all these genes have a complex expression pattern associated with diapause that may explain the lack of differences found in this study. *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. 2015), and *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 queen already in diapause (Amsalem et al. 2015). Overall, while our data examining worker physiology matched the DGPH predictions, the gene expression data provided partial support and warrant further investigation.

The study of conserved molecular pathways (‘genetic toolkits’) that regulate development in diverse species has greatly advanced our understanding of how evolution acts to generate morphological diversity. This has proven to be a powerful tool for studying social behavior in social insects, where different pathways controlling reproduction, development and behavior in solitary insects have evolved to regulate complex social behaviors (Toth and Robinson 2007).

For example, genetic toolkits regulating maternal behavior in solitary insects have evolved to regulate sibling care in eusocial species (Linksvayer and Wade 2005; West-Eberhard 1987) and genetic toolkits regulating division of labor in social species evolved from genetic pathways regulating foraging behavior in solitary species (Toth et al. 2010). The hypothesis that pathways regulating female castes evolved from pathways regulating diapause (Hunt 2007) was demonstrated in both *Polistes* (Hunt et al. 2007) and *Bombus impatiens* (current paper). All of these hypotheses are based on the same principle: evolution is often conservative and novelty can evolve by co-opting pre-existing genetic architecture (Toth and Robinson 2007). However, they are not mutually exclusive, and it is quite probable that different ecological constraints played a role in different lineages of insects, leading to similar results (social behavior) via different pathways.

One mechanism by which novelty can appear in a population is phenotypic plasticity, *i.e.*, the ability of one genotype to produce multiple phenotypes when exposed to different environmental cues (Jones and Robinson 2018; West-Eberhard 1989). Co-option of pathways regulating diapause to generate reproductive and non-reproductive phenotypes may have facilitated the evolution of the worker caste in groups such as *Bombus* or *Polistes*, or other seasonal species that were subjected to similar selective pressures. Indeed, temperatures and nutrition have been identified as the central important cues for caste determination across social insect lineages. For example, the production of discrete phenotypes in ants of temperate climates is influenced by 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). The idea that pathways regulating diapause evolved to regulate caste differences is particularly compelling in insects living in seasonal environments. Decrease in temperatures during periods of cooling may have served as a bottleneck, allowing the survival of females that were more adapted to cold temperatures (*i.e*., heavier, larger, able to store more fat-body reserves and tolerate cold).

Both the physiological and genetic mechanisms regulating diapause were shown to be conserved across species, suggesting diapause, and particularly genes regulating nutrient storage, development, and stress resistance (Amsalem et al. 2015; Denlinger 2002) were subjected to directional selective pressures (Amsalem et al. 2015; Ragland et al. 2010; Ragland and Keep

2017). Although the set of environmental conditions that facilitated caste divergence were not identical across different lineages of insects, the most recent reconstruction of the Vespidae (Hunt 2006; Piekarski et al. 2018) and Bombus (Hines 2008) phylogenies suggest that both were subjected to extensive period of cooling. Bombus, for example, are assumed to have radiated during the Miocene (Hines 2008; Prokop et al. 2017), an epoch associated with gradual cooling and a series of ice ages, that could potentially shaped their social behavior.

Conclusions

How morphologically distinct female castes evolved is a longstanding question in social evolution of insects. Increasing evidence suggests that diapause may have played an important role in the transition from solitary to social life (Hunt et al. 2007; Santos Priscila Karla et al. 2019), but empirical evidence in support of this transition are lacking. By comparing early- and late-season workers for the presence of reproductive and diapause-like signatures, we found substantial support for the DGPH in *Bombus impatiens*. Future studies examining how seasonality affects larval development and manipulation of nutritional cues in larvae could further elucidate the importance of diapause-inducing cues in early life and 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.

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Captions to figures

Figure 1: The effect of colony's age on *B. impatiens* worker oocyte size. Workers were sampled from two colonies during weeks 3-13 (calculated from the emergence of the first worker) and immediately frozen (n=472). Numbers in columns represent the number of individuals and letters in bars denote statistical differences at α=0.05.

Figure 2: The effect of colony's age on the cumulative number of eggs laid by queen-less *B. impatiens* workers. Newly emerged workers were sampled from four colonies during weeks 2-11 (calculated from the emergence of the first worker) and kept in pairs for 10 days (n=211 cages). Numbers in columns represent the number of cages and letters above bars denote statistical differences at α=0.05.

Figure 3: The effect of colony's age on *B. impatiens* worker survival in diapause-like conditions (3-5°C, 70% humidity). Group of 10 workers were sampled during weeks 3-13 from 6 colonies (n=2,417). Numbers in brackets represent the total number of workers sampled per week. Statistical significance was accepted at α=0.05.

Figure 4: The effect of colony's age on *B. impatiens* worker head width size (A) and body-mass (B). Workers were randomly sampled from two colonies during weeks 3-13 (calculated from the emergence of the first worker, n=472). Numbers in columns represent the number of cages and letters above bars denote statistical differences at α=0.05.

Figure 5: The effect of colony's age on the average percentage of fat body lipids in *B. impatiens* worker's abdomens. Workers were sampled from four colonies during weeks 3-5 (early-season) versus weeks 8-11 (late-season) (n=90). Numbers in columns represent the numbers of individuals and different letters above bars denote statistical significance at α=0.05.

Figure 6: The effect of colony's age on the expression levels of several candidate genes in 3-day old *B. impatiens* workers. Workers were sampled from 6 colonies during weeks 2 and 10 (n=17). Different letters above bars denote statistical significance at α=0.05.

Table 1: The list of variables examined in this study, the predictions stemming from Hunt's theory and whether they correspond to the findings in this study.

VARIABLE COMPARED THROUGHOUT THE COLONY DEVELOPMENT		ATTRIBUTED FUNCTION	PREDICTION ACCORDING TO THE DGPH (HUNT 2006)	RESULTS OF THIS STUDY	RESULTS MATCH PREDICTIONS?
PHYSIOLOGY	Oocyte size	Workers with a higher reproductive potential exhibit higher oocyte size	G1 pathway, oocyte size is higher in early-season ("reproduce now")	Smaller oocyte size in late-season workers	Yes
	Egg laying	Workers with higher reproductive output lay more eggs	G1 pathway, eggs laid are higher in early-season ("reproduce now")	Lower number of eggs laid in late-season workers	Yes
	Cold tolerance	Workers more adapted to diapause conditions exhibit higher cold tolerance	G2 pathway, cold tolerance is higher in late-season ("reproduce later")	Higher cold tolerance in late-season workers	Yes
	Body size	Workers more adapted to diapause conditions are larger	G2 pathway, body size is higher in late-season ("reproduce later")	Larger body size in late-season workers	Yes
	Body mass	Workers more adapted to diapause conditions are heavier	G2 pathway, body mass is higher in late-season ("reproduce later")	Larger body mass in later season workers	Yes
	Fat body lipids	Workers more adapted to diapause conditions have higher fat storage	G2 pathway, lipids are higher in late-season ("reproduce later")	Higher fat storage in late-season workers	Yes
GENE EXPRESSION	*vitellogenin* levels	Positively associated with female fertility. Reproductive females show higher *vg* expression compared with non-reproductives	G1 pathway, *vg* is higher in early-season ("reproduce now")	No differences	No differences
	mfe levels	Positively associated with female fertility. Reproductive workers have higher JH levels compared with non reproductives	G1 pathway, JH is higher in early-season ("reproduce now")	Higher in early-season workers	Yes
	hex1 levels	Encodes to storage proteins that accumulate in individuals during diapause	G2 pathway, fat storage is higher in late-season ("reproduce later")	Higher in late-season workers	Yes
	hsp70 levels	Encodes to heat shock protein that is higher in individuals during diapause	G2 pathway, cold tolerance is higher in late-season ("reproduce later")	No differences	No differences
	menin levels	A regulator of heat shock proteins and a general stress response, higher in individuals during diapause	G2 pathway, stress resistance and cold tolerance are higher in late-season ("reproduce later")	Higher in early-season workers	Opposite trend
	foxo levels	A transcriptional factor in the insulin pathway, lower during diapause	G2 pathway, metabolism is lower in late-season ("reproduce later")	No differences	No differences
	pepck levels	A gene coding to a metabolic enzyme used during gluconeogenesis. Reduced gluconeogenesis and a conservation of energy stores is typical to individuals during diapause	G2 pathway, lipids higher in late-season ("reproduce later")	Higher in early-season workers	Yes

Figure 1

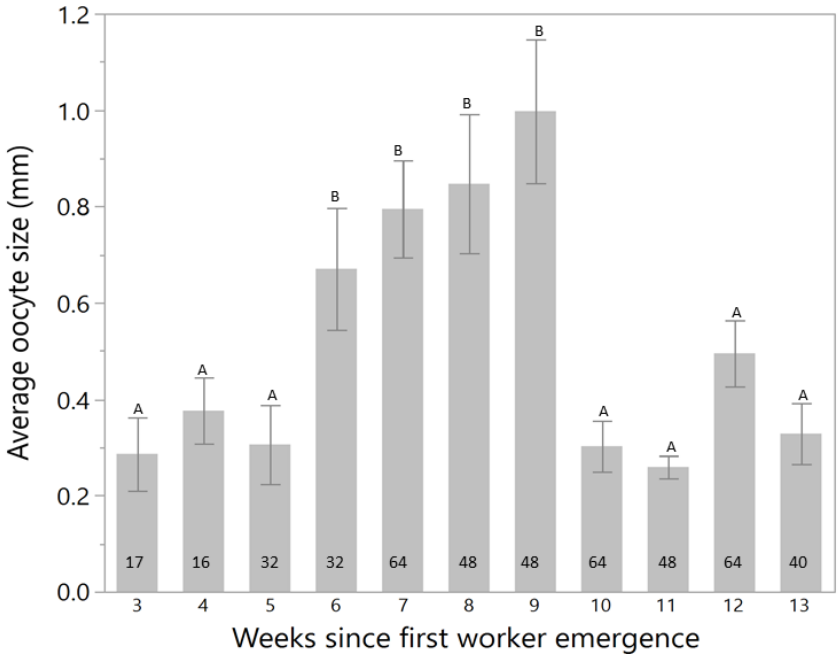

Average oocyte size (mm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
A
A
B
B
B
B
A
A
A
A
17
16
32
32
64
48
48
64
48
64
40
3
4
5
6
7
8
9
10
11
12
13
Weeks since first worker emergence

Figure 2

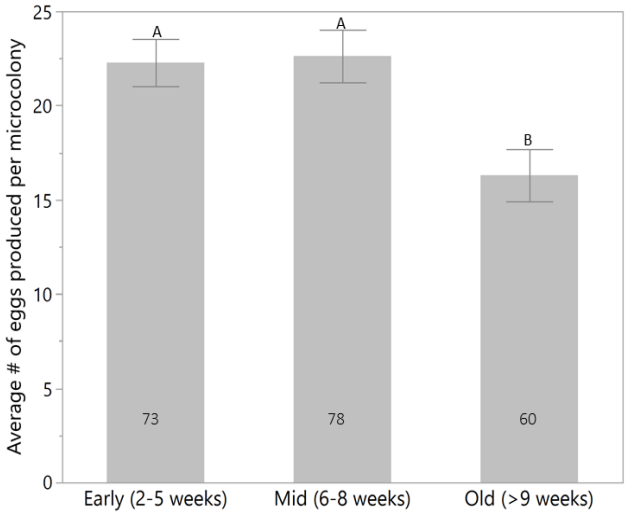

Average # of eggs produced per microcolony
25
20
15
10
5
0
A
A
B
73
78
60
Early (2-5 weeks)
Mid (6-8 weeks)
Old (>9 weeks)

Figure 3

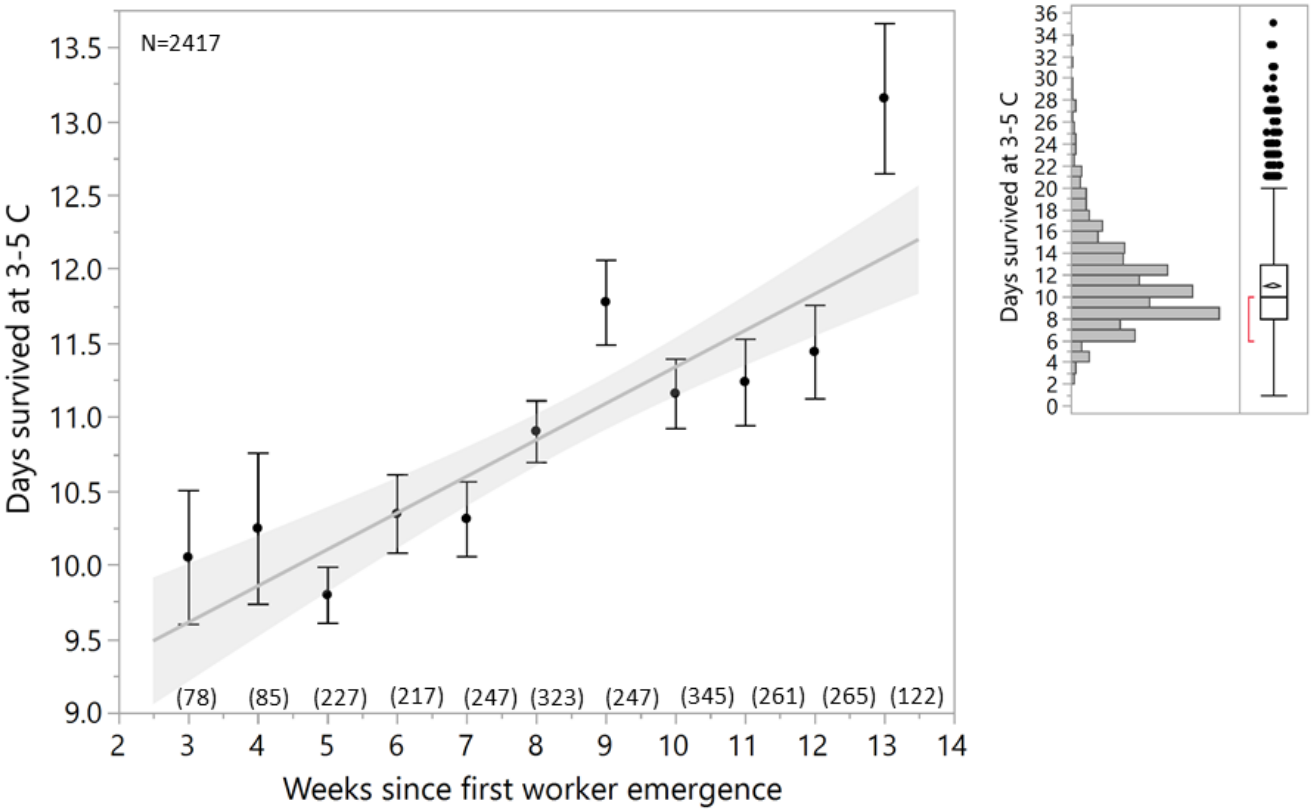

N=2417
Days survived at 3-5 C
Weeks since first worker emergence
(78) (85) (227) (217) (247) (323) (247) (345) (261) (265) (122)
Days survived at 3-5 C

Figure 4

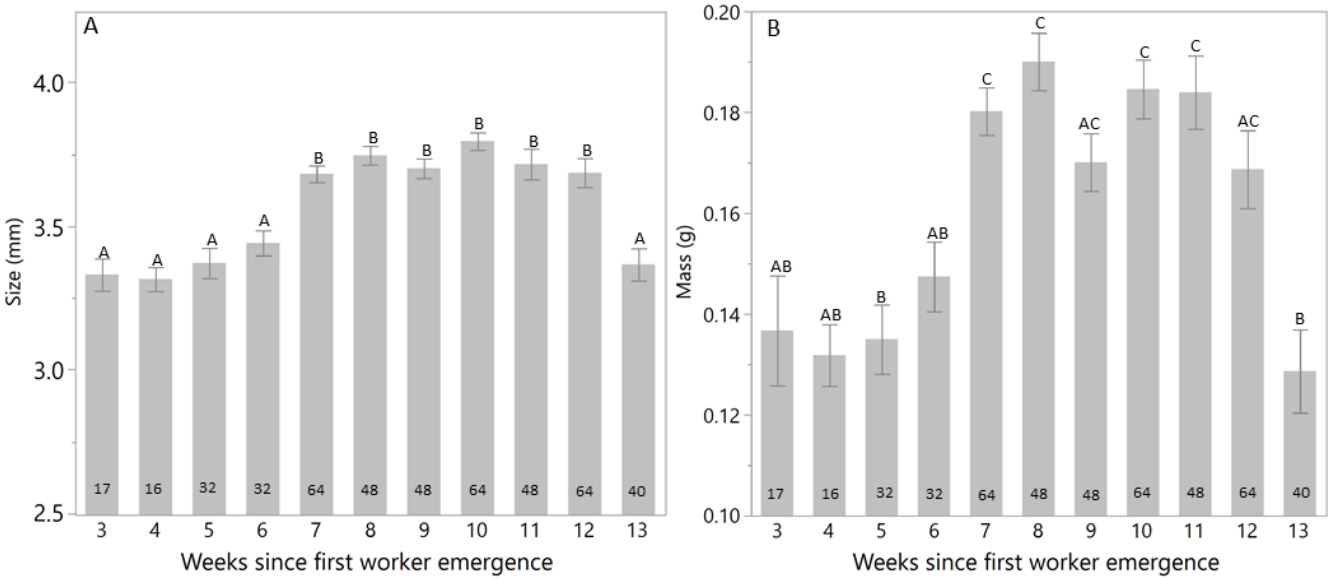

A
Size (mm)
4.0
3.5
3.0
2.5
A A A A B B B B B B A
17 16 32 32 64 48 48 64 48 64 40
3 4 5 6 7 8 9 10 11 12 13
Weeks since first worker emergence
B
Mass (g)
0.20
0.18
0.16
0.14
0.12
0.10
AB AB B AB C C AC C C AC B
17 16 32 32 64 48 48 64 48 64 40
3 4 5 6 7 8 9 10 11 12 13
Weeks since first worker emergence

Figure 5

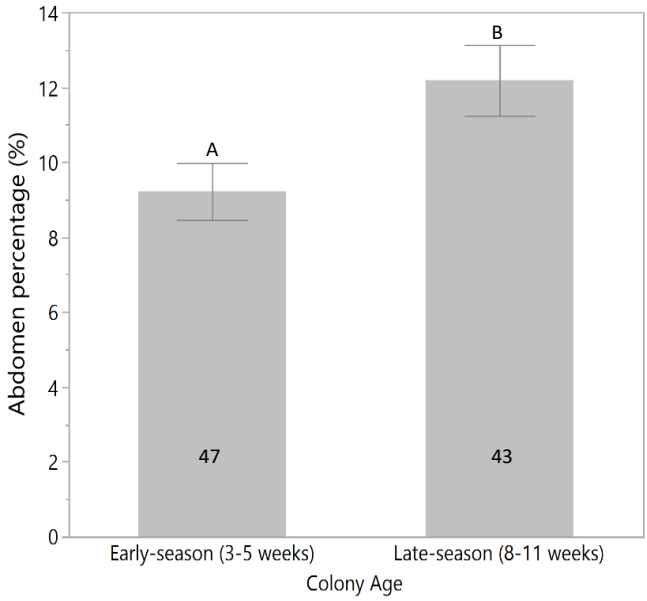
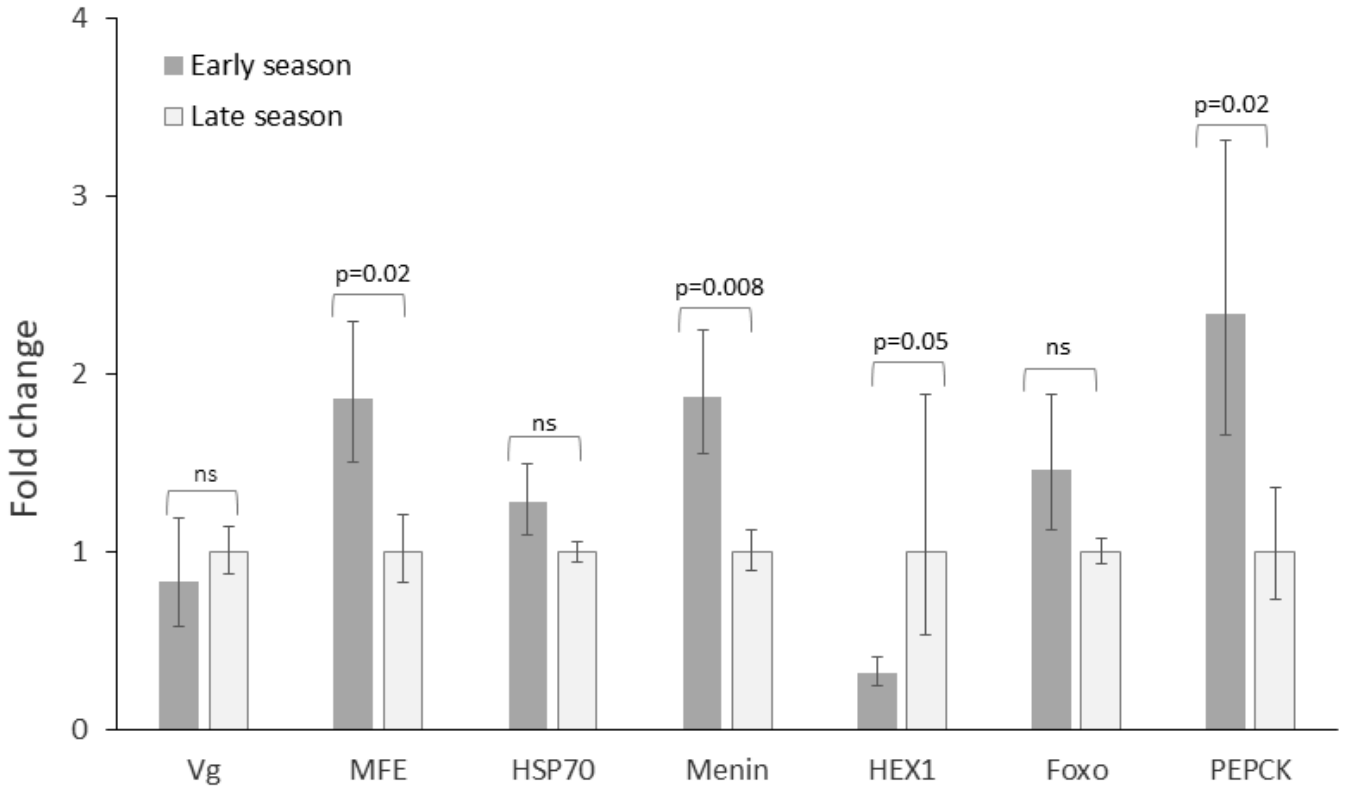

14
12
10
8
6
4
2
0
Abdomen percentage (%)
A
B
47
43
Early-season (3-5 weeks)
Late-season (8-11 weeks)
Colony Age

Figure 6


Early season
Late season
Fold change
4
3
2
1
0
ns
p=0.02
ns
p=0.008
p=0.05
ns
p=0.02
Vg
MFE
HSP70
Menin
HEX1
Foxo
PEPCK