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UNIVERSITY OF MISSOURI-ST. LOUIS
Department of Biology
Graduate Program in Ecology, Evolution, and Systematics

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B.S. Biology, Universidad Nacional de Colombia, 2004

**The effect of photoperiod on regulation of key components of the life cycle in the
bumble bee *Bombus impatiens* L. (Hymenoptera: Apidae).**

A Thesis Submitted to The Graduate School of the University of Missouri – St. Louis in
partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biology

April 2012

Advisory Committee

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Abstract

Photoperiod, the cyclical changes in day length due to earth's rotation, is a key environmental cue that organisms use directly or indirectly to regulate many aspects of their biology. In this study, I investigated whether photoperiod is sufficient to trigger changes in varied internal characteristics of colonies of the temperate bumble bee *Bombus impatiens*. In particular, I documented patterns and changes of demographic parameters during the colony's life cycle, reproduction, and social behavior.

In chapter one, I examined the effect of different photoperiod regimes on key parameters of colony development in *B. impatiens* such as colony growth, brood survival and timing for the production of reproductives. Colonies exposed to a photoperiod that simulated a natural photoperiod for temperate regions produced significantly larger colonies, reflected in higher oviposition rates and lower pupa mortality, than colonies exposed to any other photoperiod regime. Similarly, these colonies synchronized their production of reproductives, gynes (new queens) and males to the time after the longest day and subsequent decreasing of the day length. In contrast, colonies exposed to constant photoperiodic conditions of different day lengths or to photoperiods of constant increase or decrease produced reproductives at varied times during the social phase of the colony's life cycle. These results suggest that photoperiod is an important environmental cue that colonies use to regulate colony development over the social phase of the colony's life cycle, and also to synchronize the production of reproductives to match the external environmental conditions.

In chapter two, I analyzed circadian patterns of activities in colonies of the bumble bee *B. impatiens* under different photoperiod regimes. The results showed that colonies exhibit circadian characteristics similar to individual circadian rhythms. Photoperiod was sufficient to entrain circadian patterns of activity at the colony level. Colonies under constant dark conditions free run with a period close to 24h, and colonies were relatively arrhythmic under constant light regimes. Similarly, colonies sleep patterns exhibited circadian rhythmicity. Results from these experiments showed that large bees, which tend to become foragers, maintain long bouts of sleep with clear spatial preferences for the marginal areas of the colony. On the other hand, small bees have short sleep bouts that occur closer to the brood than large bees. These results suggest that colonies exhibit endogenous circadian patterns of activity and rest that could affect diverse aspects of task allocation and social development for this species.

Finally, in chapter three, I examined behavioral changes in colonies of *B. impatiens* exposed to three different photoperiod regimes. Colonies maintained a constant proportion of functional tasks that was independent of the exposed photoperiod over the social phase of the colony's life cycle. However, I found changes in the frequency for four individual behaviors as an effect of photoperiod. Additionally, I found that over the course of the colony's social phase, colonies modified the likelihood of body size classes performing particular behaviors as an effect of photoperiod, and colony age. These results suggest that colonies maintain social homeostasis by means of behavioral flexibility. Ultimately, colonies appear to be constantly assessing external environmental cues to

regulate intrinsic aspects of the colony's development and social behavior. This research therefore provides insights on the behavioral mechanisms of social regulation in relation to environmental information in *B. impatiens*.

Puff

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CHAPTER 1 - Photoperiod regulates colony development and colony reproduction in the temperate bumble bee *Bombus impatiens* L. (Hymenoptera:Apidae).

Abstract

I compared developmental parameters and timing for production of reproductives of colonies of the bumble bee *Bombus impatiens* that were exposed to eight different photoperiod regimes. Oviposition rates, larval and pupal survival, and colony growth rates were higher in colonies exposed to a seasonal photoperiod that simulates a temperate annual day length cycle than in colonies exposed to constant photoperiods over the social phase of the colony's life cycle. While larval mortality was the highest in colonies exposed to a simulated seasonal photoperiod treatment, pupal mortality was lowest from all photoperiod treatments. There was no effect of photoperiod on the mean colony's age as well as on the queen's survival. Colonies exposed to a seasonal photoperiod treatment produced gynes and males only after the longest day of the experiment and the subsequent decreasing in day length. On the other hand, colonies exposed to constant photoperiods produced gynes and males at any time over the duration of the experiment. I showed that photoperiod is a sufficient cue that can trigger changes in colony development by adjusting key demographic parameters as well as affecting within-colony timing for reproduction.

Key words: Bombus impatiens, photoperiod, colony development, production of reproductives, regulation of reproduction.

INTRODUCTION

To be synchronized with the external environment is a challenge for all individuals and populations (Dunson and Travis 1991; Hunter and Price 1992; Bradshaw and Holzapfel 2007). Environmental predictability is an important aspect of this synchronization (Nelson et al. 1990). To predict changes in environment, individuals use more or less constant periodic processes such as day-night cycles, temperature fluctuations, seasons, winds, currents, predator-prey cycles, and migrations (Devlin and Kay 2001). In many organisms, reproductive and physiological processes are regulated by photoperiodic information (Goldman 2001; Saunders et al. 2004). Photoperiodic information not only allows individuals to coordinate their metabolic responses with the external environment but also to coordinate life cycle transitions with other members of the species. These coordinated responses are likely to enhance an individual's fitness (Sharma 2003; Michael et al. 2003).

In solitary species, the relationship between photoperiod and organismal changes such as metabolism, behavior or natural history are measured at the individual and population levels (Saunders 2002). Organisms living in social groups also need mechanisms to anticipate periodic changes in the environment and adjust social activities accordingly (Moritz and Safoski 1991). Group synchronization could be regulated by social or chemical cues such as pheromonal control (Moritz and Sakofski 1991; Bloch 2009), physical contact (Southwick and Moritz 1987), auditory signals (Davidson and Menaker 2003), or olfactory signals (Levine et al. 2002). Responses to photoperiod are an additional means of social group synchronization.

Social insects that inhabit temperate regions can be an informative system to study colony-level photoperiodic responses. These species maintain tight synchrony between the colony life cycle and annual changes in temperature, daylight, and resource availability. Detailed information exists about life cycle, social organization and colony development of many temperate social insect species (e.g., Wilson 1971; Michener 1974; Brian 1983; Cameron 1989; Pamilo 1991; Robinson et al. 2005). For example, in temperate paper wasps, a change in photoperiod from shorter to longer days influences the production of males and rearing of future foundresses (Mead et al. 1990; Suzuki 1981). Temperature and photoperiod influence sex ratio and colony size in primitively social halictine bees (Yanega 1993) and brood cell size and caste in the Oriental hornet *Vespa orientalis* (Ishay et al. 1983). In Neotropical stingless bees, disruption of photoperiod produces changes in task-related behaviors (Oda et al. 2007).

In the case of bumble bees (*Bombus*), the internal environment of nests is one of constant darkness and constant temperature; therefore individuals within the nest perform all in-nest tasks without light. Foragers are the only individuals exposed to sunlight and, therefore, exposed directly to photoperiod cues (Tasei & Aupinel 1994). Previous experimental investigations of colony development and task-correlated behaviors have been pursued without explicit study of photoperiod effects (e.g., Cameron 1989; Bloch and Hefetz 1999; Baer and Schmid-Hempel 2003).

There exist about 250 species of *Bombus* most of which occur in temperate regions (Williams 1998; Cameron et al. 2007; Hines 2008). The life cycle of bumble bees can be generalized as follows, with the caveat that there exist differences across species particularly regarding the timing of each phase of the life cycle during the season, and

some tropical species may bypass diapause and the solitary phase altogether (Zucchi 1970; Cameron and Jost 1998; Taylor and Cameron 2003). The life history of bumble bees incorporates a solitary phase of an annual cycle when a new, mated queen enters diapause in late fall and then initiates a new colony in the spring. During this solitary phase of the life cycle, the queen performs all tasks, including oviposition and foraging. In the ensuing social phase, after the first worker brood emerges, workers perform the majority of tasks, with the queen performing mostly reproductive labors, including egg cell construction, oviposition, and pupal cocoon incubation. Finally, at the end of the social growth phase, the colony enters the social reproductive phase, producing young queens (gynes) and males (Sladen 1912; Alford 1975; Michener 1974).

Although the timing for each of the afore mentioned events in the life cycle is species specific, there is still great variation within each species in the precise timing and the type of event (e.g., whether a colony produces males only, gynes only, both, or none) that likely reflects intrinsic differences between colonies (Plowright and Lavery 1984; Lavery and Plowright 1985). The proximate mechanisms regulating the timing of the switch from production of workers to production of males and gynes, and in some species the beginning of the competition phase (increase in aggression levels among members of the colony over male production), are still unclear, although recent work in *B. terrestris* suggests that some of these colony decisions are controlled by the queen with some worker regulation (Alaux et al. 2005; 2006). However, many of these studies fail to identify predictor variables that help explain the variability in the timing of events within colonies raised under similar environmental conditions. Because the life cycle can be replicated under controlled laboratory conditions in a constant environment, most

research has focused on investigating intrinsic social regulators of the life cycle, assuming that the external environment has little or no effect on patterns of colony growth or colony reproductive decisions, therefore most research on life cycle in *Bombus* has been pursued while keeping the external environment constant (Alaux et al. 2005). Only a few studies have tested the role of external environmental factors such as temperature and queen photoperiod exposure, particularly during the solitary phase of the life cycle, in some aspects of colony development (e.g., Duchateau 1991; Yoon et al. 2002; Yoon et al. 2003; Amin et al. 2007a,b). Therefore, I planned and executed a suite of studies designed to more fully understand the role that the external environment plays in the regulation of colony development in colonies of bumble bees.

To address this question I selected the bumble bee *Bombus impatiens*. *B. impatiens* is widely distributed in the Nearctic Region, and it is easily found throughout its native range from the eastern region of Canada to the southern United States and east of the Rocky Mountains (Kearns and Thomson 2001). The recent development of *B. impatiens* colonies for commercial pollination in Eastern North America allows these colonies to be easily available for research (Cnaani et al. 2002). Although *B. impatiens* social behavior and colony development have not been studied as extensively as its European counterpart, *B. terrestris*, recent studies describe various aspects of *B. impatiens* colony development, social structure, and size-based task-partitioning (Cnaani et al. 2000; Jandt and Dornhaus 2009; Couvillon et al. 2010).

In my research I exposed young colonies of bumble bees to different photoperiod regimes that were continued throughout the entire colony life cycle, and I evaluated the effects of the different light treatments on a variety of colony fitness components such as

colony growth, brood survival, and production of reproductives. The central question of my research was to ask whether and how a social insect colony uses a specific environmental cue, photoperiod, to regulate and time each stage in its life cycle. My specific research objectives were to investigate (1) whether differences in photoperiod affect colony size and colony growth, (2) whether differences in photoperiod affect queen oviposition and brood survival rates, and (3) whether differences in photoperiod affect the reproductivity of colonies as reflected in the timing of production of males and gynes.

MATERIALS AND METHODS

Commercial *B. impatiens* colonies were obtained from Koppert Biological Systems. Each colony consisted of one queen, a few workers, and brood at various stages of development (Koppert colony type C). Each colony was transferred from its transportation box from the supplier to a wooden observation nest-box with a clear plastic top and a dark removable cover that blocked the entrance of light to the nest-box, thus simulating natural darkness conditions. The nest-box was composed of a main chamber (25x25x15 cm) where the colony was placed, and a smaller chamber (8x25x15 cm) where individuals transitioned between the nest and the foraging area which was used by the bees for defecation. The foraging area consisted of a mesh cage on a wooden frame (75x100x75 cm), and it was connected to the nest box with a 25x3 cm transparent plastic tube that the bees could walk through (Figure 1).

All colonies were fed fresh pollen supplied from local apiaries and honey water solution provided by Koppert (methodology according to Plowright and Jay 1966; Cameron 1989; Cnaani et al. 2000). Pollen grains were ground and mixed with honey

water solution to produce a homogeneous paste which was added directly to the colony box. Pollen was added daily but with a randomized time for feeding to prevent the colony from becoming synchronized to a feeding schedule. To avoid starvation or over-feeding, the amount of pollen was normalized to the progression of colony growth (Evans et al. 2007). The honey water solution was provided once a day in the foraging area using 10 ml transparent plastic vials hung from the roof of the foraging area. Each vial had from two to four small holes at the base from which the workers were able to extract the solution. Honey water volume was also adjusted daily according to colony growth.

Experimental Design

In order to test the effect of photoperiod on colony development of *B. impatiens*, eight (8) different photoperiod treatments were applied to 69 colonies (n = 8 or 9 colonies per photoperiod treatment). Colonies were maintained under constant temperature (28C) and humidity (50% relative humidity) (Duchateau and Velthuis 1988) in 3 isolated rooms at the Animal Care Facility at the University of Missouri-St. Louis. Three photoperiod treatments, randomly selected, were performed during a given session. I carried out a total of three sessions from 2008 to 2010, each session lasting about 4 months.

Treatments were of two types (in hours of exposure, Light:Dark [L:D]): **Five constant L:D treatments** ((1) 24 dark, (2) 8:16, (3) 12:12, (4)16:8, and (5) 24 light), and **three changing L:D treatments** ((6) steady increase in day length, (7) steady decrease in day length, and (8) a simulated seasonal photoperiod). For the simulated natural photoperiod I used annual values similar to the St. Louis region where the maximum total light is 14h52min of light on the longest day of the year. Steady increase in day length

began at 12 h 45 min light followed by an increase of 15 minutes every 5 days for the duration of the experiment. Steady decrease in day length began at 17h 15 min initial day length and decreased by 15 minutes every 5 days. Simulated seasonal photoperiod treatment began at 12 h 45 min total light (approximating the beginning of the social phase in the colony life cycle), and day length was increased by 15 minutes every fifth day until reaching a maximum of 15 hours of light followed by a decrease of day length until the end of the experiment (Figure 2).

Constant photoperiod treatments, ranging in day length from 0 to 24 hours, tested a threshold hypothesis, which assumes that there exists a day length above which significant changes in colony growth can be measured. Changing photoperiod schedules tested whether the transition from short to long nights (simulating the transition from summer to winter) is sufficient to regulate colony growth and the timing of production of reproductives or, alternatively, if an increase in night length alone or increase in day length alone is sufficient to trigger production of queens and males.

Colony Growth

To quantify colony growth I recorded the total number of egg-cells, larval-cells, pupae and adults every fifth day from the time when colonies were moved to the observation boxes to the death of the queen marking the end of the experiment. Nest boxes were not exposed to white light, with observations performed under dim red light thus also reducing the disturbance created by the observer (Peitsch et al. 1992; Chittka and Waser 1997). Observations were performed at random times to avoid synchronization of the colony to the disturbance created by the observer or any possible effects of exposure to red light.

To analyze growth rates I divided colony growth curves into two main components. The first component (C1) describes the increase in the colony size from the beginning of the experiment to the highest point of growth. The second component (C2) describes the decline of the colony from the highest point for growth to the death of the queen which marked the end of the experiment (Figure 2). The intrinsic growth rate for C1 and C2 was calculated for each colony by determining the slope of the linear regression of the logarithm of population size versus time (Gotelli 2001).

Insect life tables are frequently constructed using life stages instead of ages (Bellows et al. 1992). In this study I used egg-cells, larval and pupal stages to construct life tables. Adults were not used because to do so would require daily removal and marking of newly hatched adults that would create significant disturbance to the colony. All larval instars were grouped into one life stage (larva) in order to reduce error due to misidentification of the larval instars. Life table parameters were calculated for each colony in each treatment. Fecundity parameters counted as number of worker offspring per female were not calculated because in eusocial hymenopteran colonies most of the individuals (workers) produced in the colony will not reproduce; therefore, their contribution to the next generation is an indirect contribution that cannot be measured in terms of number of females produced per individual (Wilson 1971; Bourke 1988). To estimate the effect of photoperiod on survivorship I used the two most commonly used parameters: the standardized survival schedule $l(x)$, and the probability of survival from one stage class to the next stage class or age-specific survivorship $g(x)$ (Gotelli 2001).

Timing of the production of new reproductives

Production of reproductives was recorded as the day when the first adult male and the first queen pupae cell appeared in each colony. I then calculated the day of oviposition using the developmental times reported for the species in Cnaani et al. 2002 (22 days for queens, from egg to pupae, and 24 days for males, from egg to adult). Male brood resembles worker brood in size (worker size is highly variable in this species); therefore, I used the day at which the first male was detected in the colony as a proxy for the timing when the male eggs were laid. I did not distinguish between males produced by the queen and males produced by workers, nor did I calculate male abundance as it would have required further disturbance to the colony. New gynes are easily recognized at the late larval stages due to their large size in comparison to worker brood. I recorded the time at which the first gyne larvae appeared and the number of gynes in each colony for each treatment.

Statistical Analysis

The effect of photoperiod on colony growth was analyzed using a general estimation equation model (GEE poisson) regression with a log-link function allowing analysis of repeated measurement data that do not meet normality and homogeneity of variance assumptions. Variance parameters of time variables in a longitudinal analysis are not independent, and thus I used an AR-1 correlation structure of the data allowing higher variance correlation among closer time points (Zuur et al. 2009). Fitting of longitudinal models was done in R (R Development Core Team 2009).

Growth rate values and life table parameters were compared among the different photoperiod treatments using one way ANOVA with a Tukey posthoc HSD analysis. When normality or homogeneity of variance assumptions were not met, I used the Kruskal-Wallis non-parametric Analysis of Variance test.

I tested for equality in the timing for production of reproductives between the treatments using the non-parametric log-rank test in a survival analysis from the package survival (R Development Core Team 2009)

RESULTS

Egg production

There was a significant effect of photoperiod on the average rate of egg production (ANOVA $F=36.125$ $P<0.0001$). Colonies exposed to simulated seasonal photoperiod had the highest rate of egg production. Constant 24 h light and 24 h dark photoperiods had the lowest rates of egg production for all treatments (Figure 3). Over time, egg rate production grows in a similar pattern as colony growth. As with colony growth there is a small increase of egg production towards the end of the colony life cycle especially for the simulated seasonal, increase, and 12L:12D photoperiod treatments. Unlike colony growth patterns, the increase photoperiod treatment had the highest value of egg-cells (day 60) followed by a sharp decline in egg production. On the other hand, the simulated seasonal photoperiod had the highest value at day 65, but contrary to the increase photoperiod treatment, it maintained a stable rate of egg production until the end of the experiment (Figure 4).

Effect of photoperiod on colony size

The majority of colonies exposed to constant photoperiod treatments initially showed a negative colony growth rate followed by a slow positive recovery resulting in small colony sizes. At the time of queen death, the majority of colonies exposed to constant photoperiods displayed a reduction in population size relative to starting size. In contrast, simulated natural photoperiod and the increase photoperiod treatments maintained a logistic growth pattern, with relatively higher values of growth rates, and a larger population size over the course of the experiment compared to the population size at the beginning of the experiment (Figure 5). Colonies exposed to the simulated seasonal photoperiod treatment had significantly higher average population sizes at most brood age classes (Note that these values are a proportion normalized by the initial population size of each colony). The only exception was for the egg class, where the simulated seasonal photoperiod was not significantly higher than the simulated increase in photoperiod (Kruskal-Wallis multiple comparison test $P>0.05$). The mean population size of the colonies exposed to the simulated increase in photoperiod was significantly higher than that of all of the constant treatments and the simulated decrease in photoperiod for all brood age classes except the larva class, where there was not statistical difference among any of the treatments. All of the treatments with constant day lengths showed similar values of mean population size for all age classes (Figure 6; Table 1).

All treatments reached the highest population point at approximately the same time interval (69.9 ± 0.8 days (mean \pm SE)) with the exception of two treatments (24 light and 24 dark), where the time for reaching highest population time was significantly

earlier (37.81 ± 0.9 days (mean \pm SE)). Colonies exposed to the simulated seasonal photoperiod and the increase photoperiod treatments had the highest population size at the maximum point for growth compared to all other treatments (Figure 5).

Effect of photoperiod on colony growth

Colonies exposed to the simulated seasonal photoperiod treatment and the increase in photoperiod had significantly higher colony growth rates than all of the other treatments (GEE $X^2=278$, $P<0.001$). There were no statistical differences in colony growth rates among the constant treatments (GEE $X^2= 1.68$ $P= 0.1964$). The analysis of the effects of the treatments on colony growth rate through time showed that the colonies exposed to simulated natural photoperiod and increase photoperiod treatments responded significantly to changes in photoperiod after the time points (8 and 17) that correspond to days 35 to 80 from the beginning of the experiment (GEE $X^2=58.6$, $P=<0.001$) (Figure 5).

When the data were partitioned into the two growth phases C1 and C2 of the colony life cycle (Figure 2), simulated seasonal photoperiod and the simulated increase photoperiod treatments had the highest values of growth rate at the first component of population growth (C1). Colonies exposed to photoperiods with a stepwise increase in day length had higher growth rate values than colonies exposed to short days photoperiod (ANOVA $F=4.8913$ $P<0.001$). The low light LD 8:16, the simulated decrease, and the 24h light treatments had the lowest values for C1 (Figure 7a). For the second component of the colonies' growth rates (C2) there were no statistical differences among any of the treatments (ANOVA $F=1.77$ $P=0.1240$, Figure 7b).

Survivorship

There was a positive linear correlation of the standardized survival schedule $l(x)$ between the larvae stage class and the pupae stage class (Pearson's correlation coefficient, $R=0.29$, $df=60$, $P<0.0001$). However, when the data were partitioned among the different photoperiod treatments, only the increasing photoperiod treatment had a significant correlation coefficient (Pearson's correlation coefficient, $R=0.58$, $df=9$, $P<0.0171$). On the other hand, there was a weak negative correlation between larval survivorship and pupal survivorship $g(x)$ (Pearson's correlation coefficient, $R=-0.08$, $df=60$, $P<0.0266$). When the data were partitioned by treatments there was no significant correlation in any of the treatments. Colonies exposed to simulated seasonal photoperiod treatment had the lowest survival probability of an individual to arrive at the larval stage ($l(\text{larvae})$) compared to all other treatments. On the other hand, the highest survival probability of an individual to arrive to the larval stage ($l(\text{larvae})$) occurred in colonies exposed to the simulated increase photoperiod treatment (Kruskal-Wallis $X^2=6.1539$, $p<0.001$). Finally, there was no statistical difference of the survival probability of the larvae survival schedule $l(x)$ among all of the constant treatments. At the pupal stage the simulated seasonal photoperiod treatment gave the highest probability of survival $g(\text{pupae})$ (Table 2).

I found that colonies adjust brood survival depending on the external photoperiodic information. Colonies exposed to a simulated seasonal photoperiod will have lower larvae survival but higher pupae survival (41% larvae survival vs 63% pupae survival) as compared to constant conditions, increase, and decrease photoperiods, where I observed an opposite trend (65% larvae survival vs 50% pupae survival). However,

colonies exposed to a simulated seasonal photoperiod have higher oviposition rates than colonies exposed to any other photoperiodic condition, leading to a larger net production of individuals at the end of the colony cycle. The results obtained for the simulated seasonal photoperiod are similar to those found in other bumble bee species, where brood survival in the larvae stage is relatively low (about 40%) in colonies under natural conditions (Brian 1952; Sakagami 1967). The overall pattern for brood survivorship curves is similar to those found for other social insects that show a convex curve (Fukuda and Sakagami 1968; Miyano 1980). However, I found that photoperiod has a differential effect on brood survival. Colonies exposed to a simulated seasonal photoperiod showed a survivorship curve more closely resembling a curve where there is high larvae mortality relative to pupae mortality. In most of the other treatments, including simulated increase in photoperiod, survivorship curves had lower larval mortality relative to pupal mortality.

Queen survival

There was no treatment effect on queen survival (Kruskal-Wallis $X^2 = 4.46$ df=7 $P = < 0.7245$). Queens in all treatments lived about 92 ± 13.2 days (mean \pm SE). However, queen survival among colonies within each treatment also varied considerably. After queen death in some colonies, a proportion of the remaining workers continued to lay eggs (males only) and the colony continued to decline, the foraging effort decreased dramatically, and the colonies finally died (Table 3).

1. Males

Males were produced in all treatments; however, there was a significant difference among treatments in the proportion of colonies that produced males (Kruskal Wallis $X^2=16$ df=6 $P=0.0001$). A higher proportion of colonies produced males at the simulated seasonal photoperiod and the 24 hour dark treatments (5 of 6 and 9 of 9, respectively) than did colonies exposed to the simulated increase and simulated decrease photoperiod treatments (4 of 9 and 2 of 9 respectively). The timing for the production of males was different among treatments (Log-rank test, survival analysis $X^2=17$ df=6 $P=0.00912$; Table 3). The constant decrease treatment was excluded from the analysis because of the low number of colonies producing males. The simulated seasonal photoperiod and the simulated increase treatments produced the first male only after day 55 from the beginning of the experiment. On the other hand, all of the constant photoperiod treatments had a much larger variance in the timing for the production of males (ranging from day 25 to day 100) as compared to the seasonal photoperiod (ranging from day 55 to day 95) (Figure 8).

2. Gynes

A significant number of colonies produced gynes in both the seasonal photoperiod treatment and the LD 12:12 (5 of 6, and 4 of 9 respectively). There was no gyne production in the simulated increase, simulated decrease or in the 24 hour light treatments. The timing for the production of gynes was different between the simulated seasonal photoperiod and the constant LD 12:12 treatment (Log-rank test, survival

analysis $X^2=6.3$ $df=1$ $P=0.0122$). In the simulated seasonal photoperiod treatment gynes were first produced after day 55 (78.75 ± 17.9 (mean \pm SE)), whereas in the constant LD 12:12 treatment gynes were produced before day 50 (40 ± 5 (mean \pm SE)) (Figure 8).

DISCUSSION

I tested the hypothesis that colonies use photoperiodic information to adjust the timing of key events in the life cycle. I have found that photoperiod is a sufficient, but not necessary, environmental cue for *B. impatiens* colonies to modify (regulate) colony growth, brood survival, and timing for reproduction. Day-night cycles are not a required cue, because in all photoperiod treatments, I observed most of the social phases that characterize a bumble bee annual life cycle. I have shown that photoperiod plays a role as an important informational resource for the colony to synchronize its internal social phases to the environment. In these experiments I was able to show significant differences in patterns of growth and synchronization of timing for production of reproductives that are linked to photoperiod. Other studies have also shown that photoperiod is a critical environmental cue during the bumble bee life cycle, for example Amin et al. 2007 showed that post-hibernated *B. terrestris* queens exposed to short photoperiods LD 8:16 resulted in higher colony initiation rates, higher number of first brood workers, and higher rates of queen survival.

From these results it seems that colonies that at the beginning of the growth phase were exposed to photoperiods with an increase in the day length, will be stimulated to higher queen oviposition rates, whereas colonies under constant conditions independent

of the duration of the day (or night) will maintain relatively low to constant rates of oviposition (similar results were found by Duchateau and Velthuis (1988) for colonies of *B. terrestris* maintained under constant conditions). These results can explain the apparent difference in oviposition rates found in experiments where field colonies and experimental colonies are compared. In those experiments, the oviposition rates in field colonies are usually larger than those under laboratory conditions. In my experiment, colonies exposed to a simulated natural photoperiod had similar oviposition rates to those found by Duchateau and Velthuis (1988) for *B. terrestris* of about 2.1 egg cells per day (2.2 +/- 0.3 in this experiment), whereas under constant conditions the oviposition rates drop to about 1.1 egg cells made per day (I recorded 1.29 +/-0.3 egg cells per day, Alaux et al. 2005 reports 1.1 +/- 0.2 *B. terrestris*).

Brian (1965) describes a sigmoidal curve of colony growth for bumble bees. In my experiment all of the colonies, independent of photoperiod, showed similar sigmoidal curves of growth and egg laying rates. Because these colonies were purchased from a commercial company, I did not observe the initial stages of colony initiation that could potentially contribute to changes in colony growth and the timing of events in the colony life cycle. However, the patterns of growth are similar of those reported in the literature for colonies reared from wild queens, therefore suggesting no effect from the commercial rearing conditions on growth. Photoperiod had an effect especially at the exponential section of the curve where colonies with a stepwise increase in day length had higher rates of growth than colonies under constant day lengths. At the beginning of this experiment all treatments had relatively similar growth rates, after day 35 from the beginning of my experiment colonies that had been exposed to the simulated seasonal

and increase photoperiods showed significantly higher growth rates than colonies exposed to constant day lengths (Figure 4). These results are not explained by an increase in the food supply derived from the extended amounts of foraging time in longer days, because I controlled for the daily amount of food given to colonies so that they would have only the amount of food sufficient to maintain the immediate brood numbers. In other words, the amount of food given to each colony was correlated with the time-specific oviposition rate.

It has been reported for other bumble bee species, from temperate and tropical regions, that survivorship curves for adult workers under natural conditions resemble a type I curve with a relatively low mortality at younger ages (Brian 1952; Garófalo 1978; Silva-Matos and Garófalo 2000). The survivorship curves for immature workers obtained in these experiments also resemble a type I curve except for the simulated natural photoperiod treatment that exhibited a curve more similar to a type II. However, I did not measure adult worker mortality in my experiments because the workers were confined to foraging cages and I expected to obtain lower mortality rates compared to field colonies, as reported for other *Bombus* species, thus likely skewing the shape of the final survivorship curve (*B. terrestris* Shykoff and Müller 1995; *B. griseocollis* Cameron 1989; *B. lucorum* Müller and Schmid-Hempel 1992). Therefore, these results do not describe the survivorship for this species, but can be interpreted as a plastic response to survivorship at different life stages due to changes in environmental conditions in this case photoperiod.

As reported in earlier studies, I also observed great variability in terms of colony size and the timing of the social stages in the life cycle among colonies maintained under

similar conditions. For example, the average colony size is highly variable even in colonies under similar photoperiod treatments (Pomeroy and Plowright 1982; Duchateau and Veltuis 1988; Cameron 1989; Schmid-Hempel and Heeb 1991; Cnaani et al. 2000; Burns 2004; Alaux et al. 2005). These observations have led to the belief that the external seasonal environment is unlikely to be the mechanism responsible for the control or regulation of these events, but instead that they are a consequence of endogenous characteristics of the colony (Alaux et al. 2005). However, single organisms under the absence of environmental cues also exhibit most of the behavioral and physiological processes that are common for the species, but environmental signals such as photoperiod entrain the individual's internal clock to synchronize such processes to the external environment (Saunders 1997; 2002, Sharma 2003; Bradshaw and Holzapfel 2007). In social groups there could be a similar mechanism for environmental synchronicity and still be consistent with the observations of colonies maintained under constant laboratory conditions (Bloch 2009). There is evidence for a colony clock in several species of social insects (Southwick and Moritz 1987; Bloch 2010). For example, Stelzer and Chittka (2010) have shown that colonies of *B. terrestris* in the northern temperate regions of Europe maintain strong circadian rhythms with resting periods even at latitudes at 24 hours of sunlight.

Reproductives are naturally produced during late summer or fall (long night) conditions where worker production is reduced or completely stopped in some species (Sladen 1912). I show that photoperiod was not necessary for the production of males and gynes, because colonies produced reproductives in all conditions, but photoperiod is sufficient to induce changes in the timing of the production of reproductives such that the

colonies exposed to natural photoperiods constrain the production of reproductives at the decreasing phase of the photoperiod, which will correspond to the late summer and fall in nature (Figure 7). It is likely that the production of males is an independent mechanism different from the production of gynes. I base this conclusion on the fact that in my experiment all treatments produced males and the timing for the production of males was treatment dependent.

A surprising result was the observation that the decreasing photoperiod treatment had only one colony producing males and no gynes were produced in any of the colonies tested. The decreasing photoperiod simulates fall conditions where colonies normally are large enough to start producing reproductives. This suggests that colonies under photoperiodic conditions in which they would be expected to switch to produce reproductives probably need to attain determined sizes before the switch. This hypothesis needs to be further tested. Similarly, the high degree of variability in the timing for male production within colonies raised under similar conditions is consistent with the published data for other bumble bee species, where there seem to be two types of colonies, early male producers and late male producers. Some of the hypotheses proposed to explain this separation are related to kin selection and queen-worker conflicts, which are not within the scope of this paper (but see Duchateau and Velthuis 1988; Muller and Schmid-Hempel 1992; Beekman and Van Stratum 2000).

Only the simulated seasonal photoperiod and the LD 12:12 treatments produced gynes in over 40% of the colonies. However, the timing for the production of gynes between these two conditions was different. Whereas in the simulated natural photoperiod the production of gynes was limited to the phase of decreasing day-length,

under LD 12:12 conditions colonies produced gynes very early in the colony life cycle. Similarly, I found no correlation between the onset of gyne production and the switch point (the beginning of male production) (*B. terrestris* Duchateau and Velthuis 1988; *B. lucorum* Müller and Schmid-Hempel 1992). Also, there was no correlation between the worker/larvae ratios and the timing for the production of males or gynes as proposed by Plowright and Plowright (1990) or the number of workers present in the colony (Röseler 1967). I did not observe production of gynes in colonies exposed to an increased photoperiod treatment. This treatment produced larger colony size than the simulated seasonal photoperiod treatment. It seems possible that colonies use the shift from long days to short days as a cue for the initiation of the production of gynes.

B. impatiens life cycle differs from *B. terrestris* in several aspects (see Cnaani et al. 2002 for a complete review). In *B. terrestris* there is a well defined competition point where the levels of aggression, oophagy, and worker oviposition increase (Sladen 1912; Duchateau and Velthuis 1988). In contrast, for *B. impatiens* the competition point is less well defined and the time of occurrence is less consistent between colonies. As reported by Müller et al. 1992, there is a large overlap between production of reproductives and workers in both species. However, in *B. terrestris*, at the end of the life cycle colonies switch to produce males only, whereas in *B. impatiens* there is always a mix of workers and males at this stage of the life cycle. *B. impatiens* has been reported to have larger colonies and have longer life span than *B. terrestris* (Burns 2004). Similarly, the production of males and queens occurs over a much longer time span in *B. impatiens* colonies. Finally, aggression levels between workers and the queen of *B. impatiens* are much lower than those of *B. terrestris*. These observations suggest that there are

differences among species in the mechanisms that determine the timing for particular events in the life cycle among bumble bees. This could be relevant for hypotheses concerning kin selection and queen-workers conflict over reproduction, especially since *B. terrestris* queens mate only once whereas it has been reported that *B. impatiens* queens can mate with more than one male, thereby affecting the relatedness within the members of the colony (Cnaani et al. 2002). *Bombus impatiens* nests are constructed in nature in concealed locations. The colony chamber itself is never exposed to direct sunlight. The queen remains in the chamber devoid of any photoperiodic information after workers take over the foraging tasks. The experimental setup replicates this natural situation.

CONCLUSIONS

Results of this experiment lead to four conclusions and suggest four interpretations. 1) There is a flow of photoperiod information from the exterior to inside the colony chamber. The most likely candidates for information flow are the foragers, which could take the photoperiodic information back to the colony. 2) Colonies are able to use photoperiod as a cue to regulate stages in their life cycle. A likely candidate for such regulation would be to differentially allocate resources for growth or reproduction. 3) Under constant photoperiods there seems to be regulation of phases in the life cycle based on internal characteristics of the colony. Possible mechanisms need further investigation. 4) Under conditions of otherwise constant environmental variables, *B. impatiens* has broad plasticity of colony-level response to different photoperiod regimes. This inherent plasticity could be a significant component of local adaptation across a broad latitudinal geographic range or rapid adaptation to climate-based northward shift in

geographic range. These four conclusions collectively contribute to our understanding of how social insects regulate colony-level fitness components such as growth, survival, and reproduction using photoperiod as an environmental cue.

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Table 1. Mean population sizes for each developmental stage of colonies of the temperate Bumble bee *Bombus impatiens* exposed to different photoperiod regimes.

Numbers within parenthesis are standard deviations.

Treatment	Eggs	Larva	Pupa
Seasonal	11.32 (5.52)	15.63 (6.97)	37.06 (16.13)
Increase	8.82 (5.93)	20.14 (10.84)	38.56 (25.02)
Decrease	7.70 (4.14)	16.75 (6.94)	38.96 (25.95)
LD 8:16	6.96 (3.01)	12.63 (5.06)	36.13 (16.46)
LD 12:12	7.20 (4.06)	16.24 (7.27)	27.76 (17.54)
LD 16:8	8.67 (4.05)	15.55 (6.25)	37.12 (16.34)
24h Light	4.41 (2.38)	10.88 (5.18)	27.64 (20.42)
24h Dark	5.10 (2.94)	11.38 (6.46)	26.77 (19.89)

Table 2. Survivorship data for each stage of development in colonies of the temperate bumble bee *Bombus impatiens* exposed to different photoperiod regimes. Numbers within parenthesis represent standard deviations. S(x) total number of individuals at age x, l(x) survivorship schedule (S(x)/S(0)), g(x) survival probability or age-specific survivorship (l(x=1)/l(x)) (Gotelli 2001).

Treatment	S (eggs)	S (larvae)	S (pupae)	L (Larvae) Larvae/egg	L (Pupae) Pupae/egg	G (Larvae) l(egg)/l(larvae)	G (pupae) l(larvae)/l(pupae)
Seasonal	177.68 (28.93)	73.62 (20.16)	46.89 (11.86)	0.414 (0.084)	0.265 (0.031)	0.414 (0.084)	0.636 (0.089)
Increase	134.77 (43.19)	102.92 (30.68)	43.82 (12.76)	0.763 (0.172)	0.325 (0.127)	0.763 (0.172)	0.425 (0.098)
Decrease	123.24 (22.89)	88.47 (14.94)	34.39 (5.97)	0.717 (0.110)	0.279 (0.063)	0.717 (0.110)	0.388 (0.065)
LD 8:16	109.76 (26.32)	64.73 (13.57)	36.79 (7.07)	0.589 (0.058)	0.335 (0.076)	0.589 (0.058)	0.568 (0.102)
LD 12:12	108.58 (29.35)	63.16 (32.58)	27.63 (7.23)	0.581 (0.044)	0.254 (0.051)	0.581 (0.044)	0.437 (0.057)
LD 16:8	137.69 (15.76)	79.56 (9.97)	38.50 (3.77)	0.577 (0.057)	0.279 (0.042)	0.577 (0.057)	0.483 (0.068)
24h Light	70.99 (20.13)	55.27 (19.07)	26.73 (4.14)	0.778 (0.252)	0.376 (0.130)	0.778 (0.252)	0.483 (0.104)
24h Dark	85.94 (19.68)	49.09 (21.34)	25.74 (8.45)	0.571 (0.099)	0.299 (0.111)	0.771 (0.099)	0.524 (0.165)

Table 3. Summary of results obtained per colony of the timing for production of males and gynes, and the day at which the queen died for each photoperiod treatment.

Treatment	Colony	Start Date	Days to First Male	Days to First Gyne	Days to Queen death
Seasonal	1	8/21/2008	55	-	110
	2	8/21/2008	90	-	105
	3	8/21/2008	-	-	65
	4	8/21/2008	90	55	105
	5	8/21/2008	70	-	80
	6	8/21/2008	75	75	95
	7	6/1/2007	65	90	95
	8	6/1/2007	85	95	90
	9	6/1/2007	95	-	90
Increase	1	2/8/2009	60	-	50
	2	2/8/2009	-	-	105
	3	2/8/2009	-	-	105
	4	2/8/2009	-	-	100
	5	2/8/2009	90	-	50
	6	2/8/2009	80	-	105
	7	2/8/2009	-	-	90
	8	2/8/2009	-	-	100
	9	2/8/2009	65	-	85
Decrease	1	2/8/2009	-	-	95
	2	2/8/2009	-	-	110
	3	2/8/2009	-	-	105
	4	2/8/2009	-	-	65
	5	2/8/2009	-	-	105
	6	2/8/2009	90	-	80
	7	2/8/2009	-	-	95
	8	2/8/2009	-	-	95
	9	2/8/2009	-	-	90
LD 8:16	1	8/21/2008	85	-	105
	2	8/21/2008	65	-	80
	3	8/21/2008	60	-	105
	4	8/21/2008	-	-	85
	5	8/21/2008	35	45	95

	6	8/21/2008	40	-	75
	7	8/21/2008	-	-	70
	8	8/21/2008	25	-	105
LD 12:12	1	2/8/2009	-	40	90
	2	2/8/2009	70	-	90
	3	2/8/2009	-	-	105
	4	2/8/2009	40	-	100
	5	2/8/2009	30	-	100
	6	2/8/2009	-	35	95
	7	6/1/2007	30	-	85
	8	6/1/2007	35	50	95
LD 16:8	9	6/1/2007	50	50	90
	1	8/21/2008	25	65	110
	2	8/21/2008	-	-	105
	3	8/21/2008	100	-	65
	4	8/21/2008	-	-	105
	5	8/21/2008	95	-	80
	6	8/21/2008	-	-	95
	7	8/21/2008	65	-	95
	8	8/21/2008	-	-	90
24h Light	1	4/29/2010	-	-	90
	2	4/29/2010	-	-	105
	3	4/29/2010	45	-	110
	4	4/29/2010	35	-	105
	5	4/29/2010	-	45	100
	6	4/29/2010	30	-	100
	7	4/29/2010	50	-	100
	8	4/29/2010	30	-	95
24h Dark	1	4/29/2010	75	-	100
	2	4/29/2010	35	-	100
	3	4/29/2010	45	-	105
	4	4/29/2010	25	-	100
	5	4/29/2010	25	-	100
	6	4/29/2010	40	-	80
	7	6/1/2007	25	70	80
	8	6/1/2007	35	-	100
	9	6/1/2007	80	-	90

FIGURE LEGENDS

Figure 1. Experimental set up used to investigate the effects of photoperiod on colony development in the bumble bee *Bombus impatiens*.

Figure 2. Population growth curve for an idealized temperate bumble bee colony. C1 represents the initial colony growth component ranging from the lowest initial population size until maximum size is reached; C2 represents the colony population decline component from the maximum size point until the queen's death. Population growth is normalized by the initial population size.

Figure 3. Effect of photoperiod on the average daily egg-cells laying rate of the bumble bee *B. impatiens*. Groups with different letters are significantly different (LSD post hoc test $P < 0.001$). Data represent mean \pm and standard error of the mean.

Figure 4. Effect of photoperiod on oviposition rates over time in the bumble bee *B. impatiens*. Colonies produce significantly more eggs on average over time under the simulated seasonal photoperiod and the simulated increase photoperiod treatments than the decrease photoperiod and all of the constant photoperiod treatments.

Figure 5. Population growth (normalized by the initial population size) observed during the colony's life span. Colonies are significantly larger on average over time under the simulated seasonal photoperiod and the simulated increase photoperiod

treatments than the decrease photoperiod and all of the constant photoperiod treatments (GEE $X^2=278$, $P<0.001$).

Figure 6. Effects of photoperiod on the average population size of the Bumble bee *B. impatiens* at the different stages of development (normalized by the initial population size). Data represent mean.

Figure 7. Photoperiod treatments affect the initial population growth component (C1) but not the declining growth component (C2) in the Bumble bee *Bombus impatiens*. **A.** Initial component of colony growth (see methods), the values for population growth are normalized by the initial population size. Data represent mean \pm and standard error of the mean. **B.** Second component of population growth that represents the declining phase of the colony until the queen's death.

Figure 8. Timing for the production of males (Above) and gynes (Below) at different photoperiod treatments in the bumble bee *Bombus impatiens*. Open circles represent time point of production of the first male or gyne in each colony.

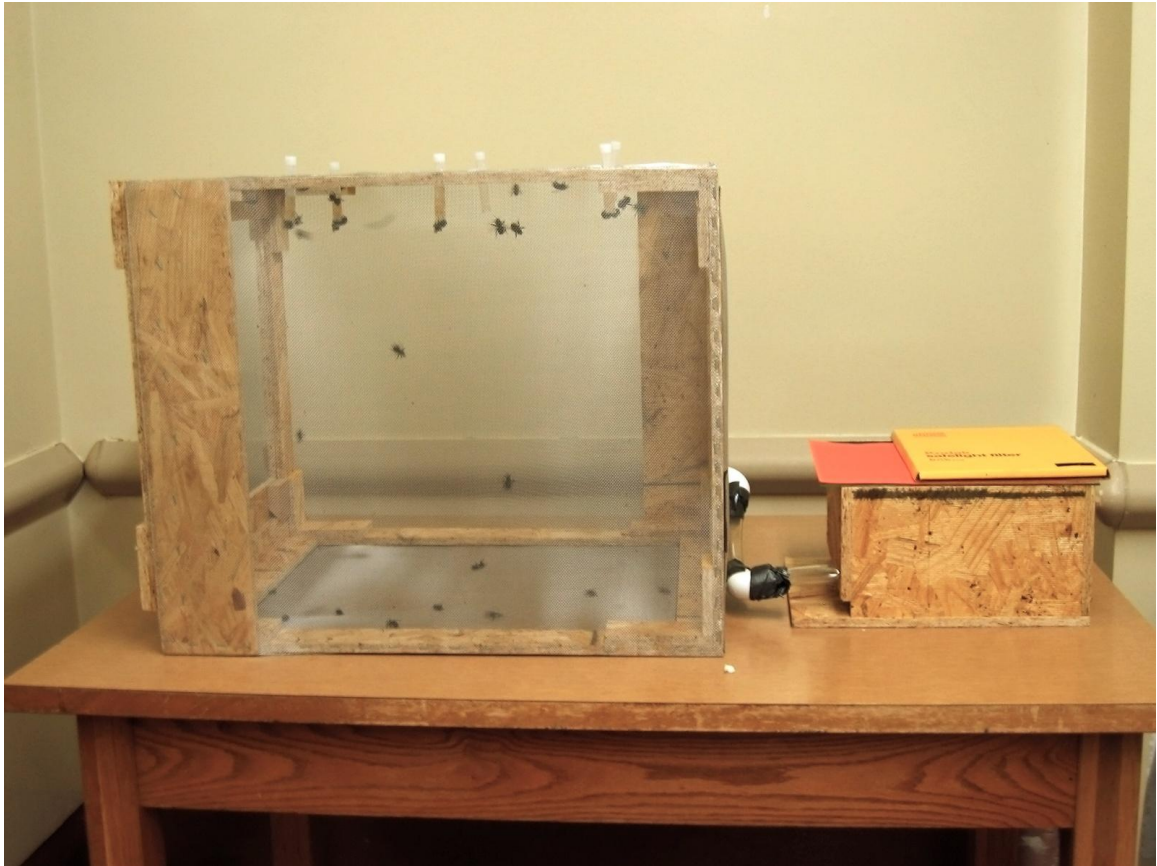


Figure 1

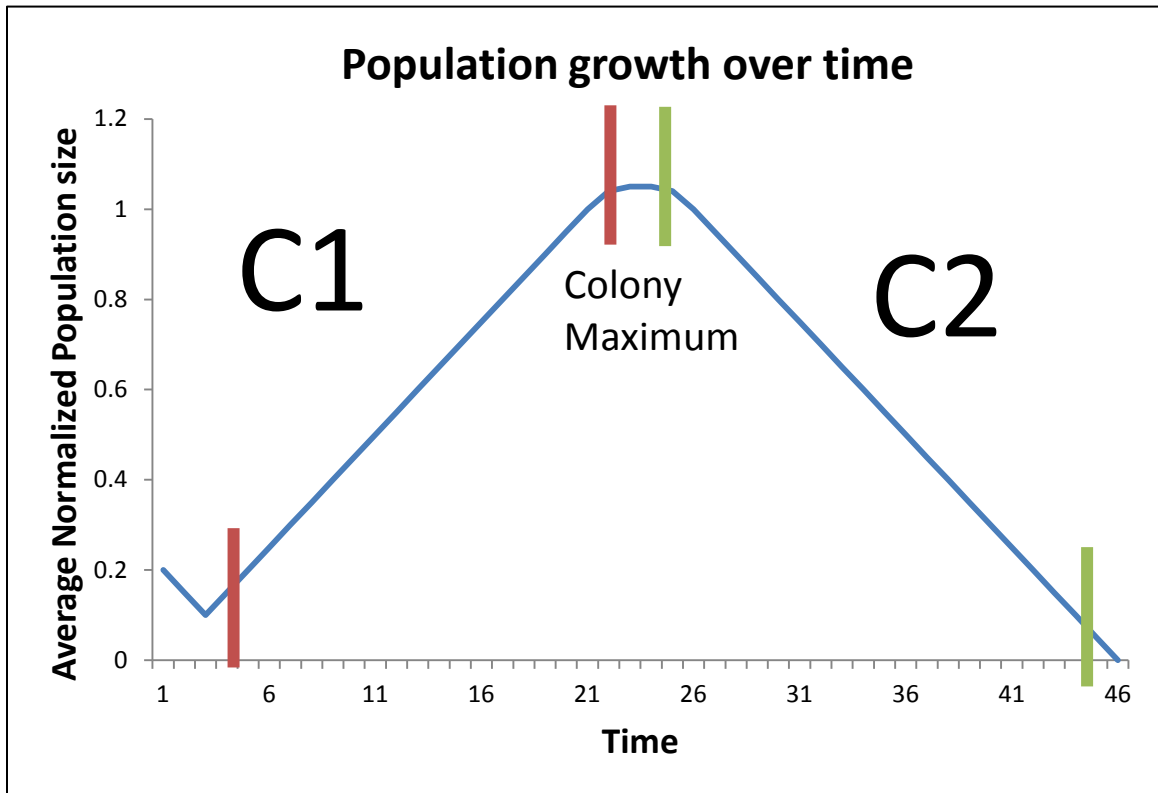


Figure 2

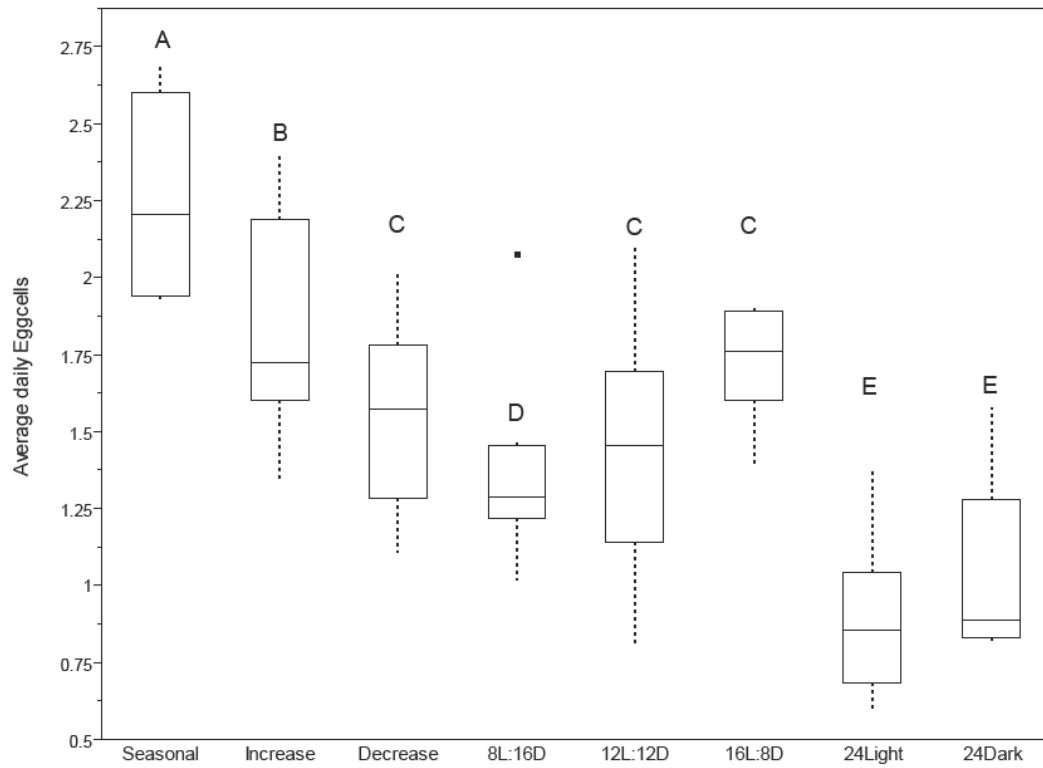


Figure 3

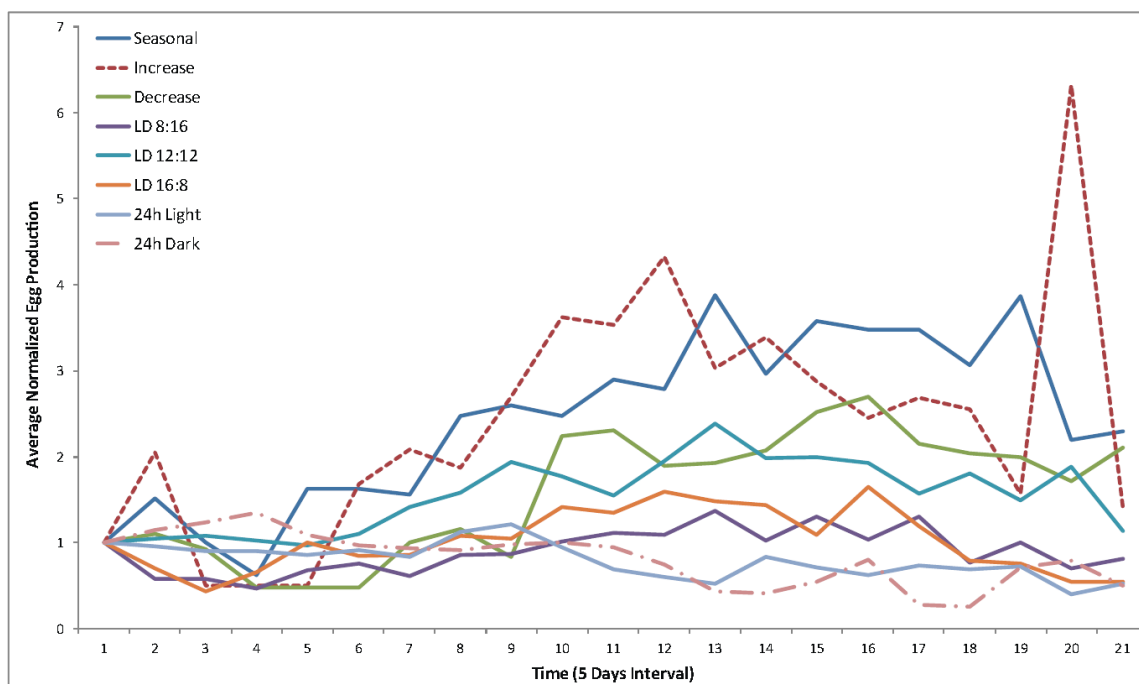


Figure 4

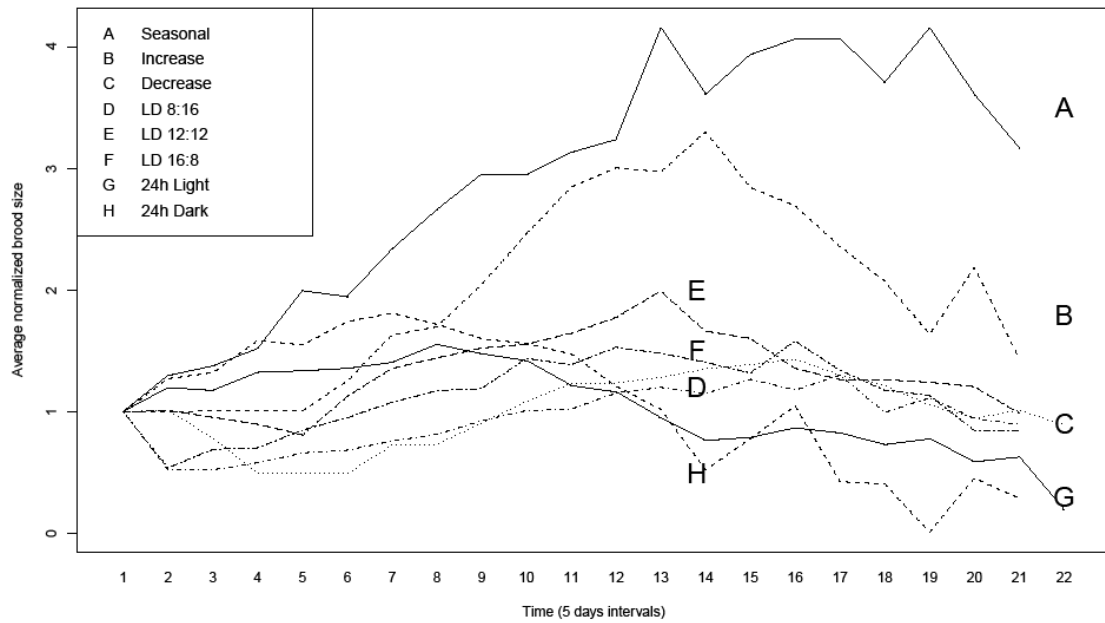


Figure 5

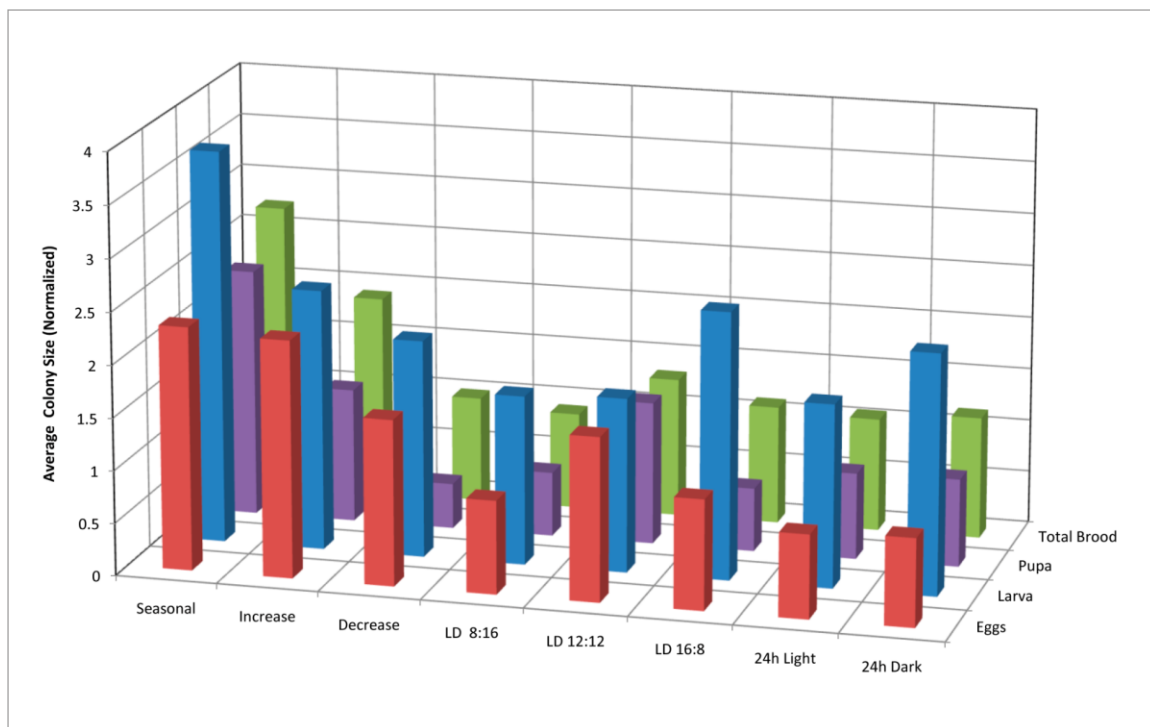


Figure 6

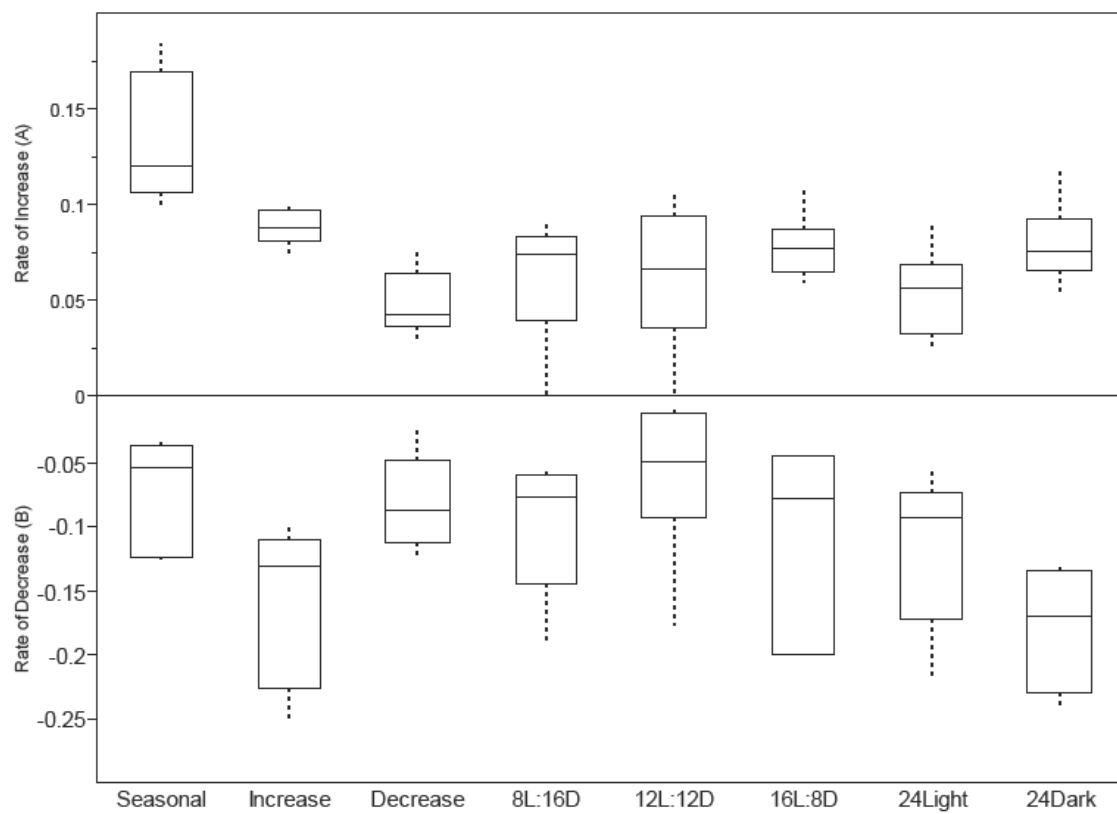


Figure 7

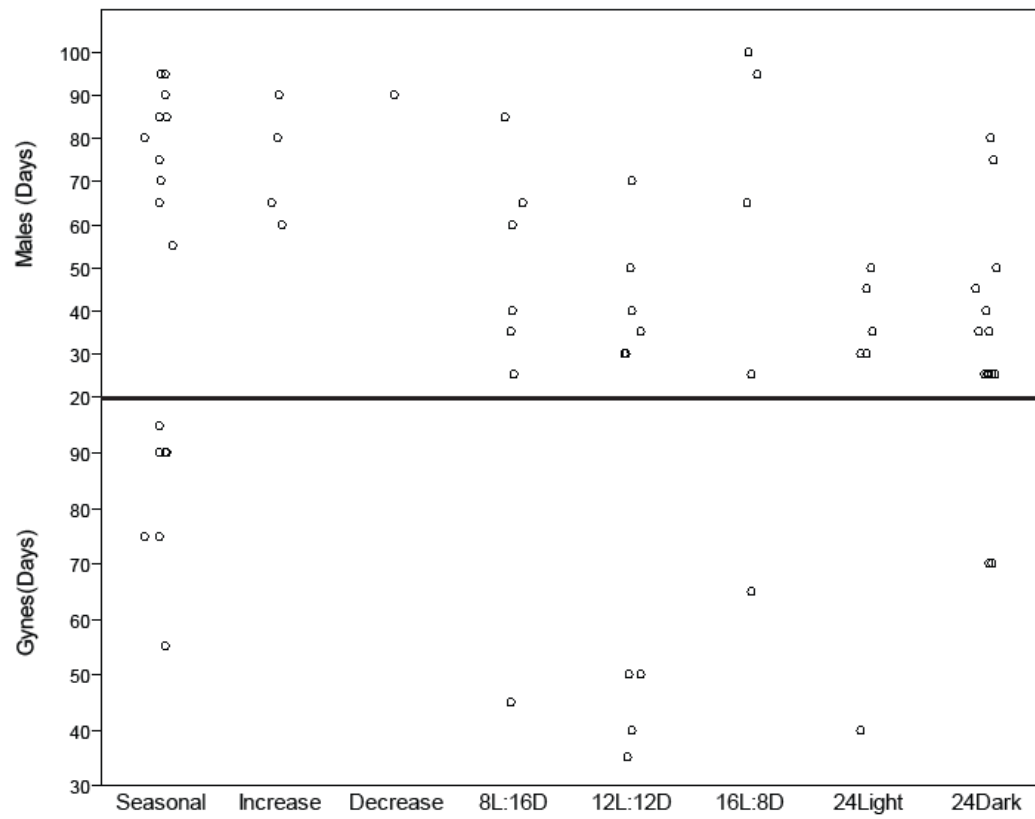


Figure 8

CHAPTER 2 - Photoperiod and circadian activity in the bumble bee *Bombus impatiens* L. (Hymenoptera: Apidae): an experimental analysis.

Abstract

I measured circadian patterns of activity and rest in laboratory maintained *Bombus impatiens* colonies exposed to four different photoperiod treatments using an electronic bee counter to continuously record worker movement activity from the nest to the foraging area. I found that colony-whole circadian levels of activity are synchronized to the exposed photoperiod schedule. Colonies under constant darkness showed evidence of an endogenous circadian rhythm with a period close to 24h that free runs in the absence of light information. Colonies were entrained to the light schedule in conditions under a seasonal photoperiod that simulates a temperate annual day length cycle, and constant 12L:12D over the social phase of the colony's life cycle. In contrast, colonies exposed to constant light schedules, showed continuous activity without an apparent daily synchronization of activity. I also measured daily cycles of rest for the different photoperiod treatments. Workers within the nest tend to sleep in larger numbers during the subjective night (scotophase) than during the subjective day (photophase) even though the nest is in constant darkness. I found a distinctive spatial segregation of rest patterns in which large bees tend to rest farther from the colony center and also farther from the queen than smaller bees. In conclusion, colony-level circadian rhythms are similar to those found in individual organisms. Colony-level circadian rhythms respond to different photoperiod treatments, thereby indicating regulation at the colony level,

Individual differences in circadian patterns of activity and rest contribute to the colony-level responses to different photoperiod treatments.

Key words: Bombus impatiens, photoperiod, circadian rhythms, sleep patterns.

INTRODUCTION

Individuals use external environmental cues to regulate life history events to predictable environmental changes. Adaptations to read these environmental cues via vision, hearing, or other senses have emerged through natural selection (Saunders 1982). Photoperiod, the cyclical change of sunlight produced by the earth's rotation around its axis, is an environmental cue that has been uninterrupted since the beginning of life (Tauber et al. 2004). In the context of this highly predictable environmental cue, an endogenous circadian clock evolved as an internal time tracking mechanism by which organisms perceive photoperiod cycles. The endogenous circadian clock is one of the most important adaptations as it operates as a regulator of both behavior and internal processes of organisms (Saunders 1982; Rosbash 1995; Denlinger 2002) including cyanobacteria and algae as well as all metazoans (Hastings and Maywood 2000; Stoleru et al. 2007; Tauber 2004). One characteristic property of the endogenous circadian clock is its adjustment or entrainment to variations in external conditions, or *Zeitgebers*, including photoperiod. Furthermore, this endogenous clock is self-sustained under conditions with no external cues, and in most organisms it oscillates close but not exactly to a 24 hour period (hence the name circadian circa=close dian=day) (Saunders 1982; Harmer et al. 2001; Dunlap et al. 2004). Extensive studies of the biological clock have been pursued in mammals (Hastings and Herzog, 2004), invertebrates (Saunders 2004) and plants (Panda et al. 2002). Some of the activity patterns used to study the nature and characteristics of circadian clocks are daily patterns of locomotor activity and periodic patterns of adult invertebrate eclosion, among others (Levine et al. 2002a).

Many fundamental activities of a social insect colony are regulated by biological rhythms (reviewed in Bloch 2009). In honey bees, for example, there is a gradual maturation of the circadian clock related to individual age (Toma et al. 2000; Bloch et al. 2002). Young individuals work around the clock taking care of the brood, and as these individuals age they begin to behave in a more circadian manner, especially during foraging when the exact timing of events leads to efficient resource exploitation (Moore et al. 1998; Shemesh et al. 2007; Bloch 2010). Honey bees are able to learn and predict daily and seasonal floral schedules, and foragers also measure time elapsed from leaving the colony to the moment of return and thereby correctly adjust their position and orient toward the nest (Bloch and Robinson 2001; Pahl 2007). The colony maintains a social synchronism of these rhythms (Moritz and Sakofski 1991; Shemesh et al. 2007), and there is an apparent caste-correlated division of clock-regulated tasks (Ben-Shahar 2005). Thus, the honey bee colony is intimately regulated by a communal circadian clock.

Bumble bees (*Bombus*) are primitively eusocial insects that provide a good system to investigate the relationship between environmental changes and temporal dynamics within social insect colonies. The life cycle of a temperate bumble bee colony is strongly linked to daily and annual seasonal changes. In the spring a mated queen will emerge from diapause and independently construct a new nest and initiate a colony. She will lay eggs and forage for food during this first, solitary stage of the life cycle. At this early stage *Bombus* queens display strong circadian rhythms of activity, but once the first brood is produced there is a weakening of the queen's circadian rhythm, and brood care is maintained around the clock until the first workers emerge (Eban-Rothschild et al. 2011). The now multi-female colony moves into a social growth phase with continuous

production of workers from queen-laid eggs. Strong caste-dependent division of circadian activities is prominent during this social growth phase in which small individuals are more likely to be in-nest workers with weak circadian rhythms, and large foragers show robust circadian activity patterns (Brian 1952; Yerushalmi et al. 2006; Bloch 2010). As the season advances the colony switches to production of new reproductive individuals (males and gynes), and this continues until the old queen and remaining workers die. Gynes (pre-queens of the next generation) and males depart the colony, mate, and newly mated queens then enter diapause (Michener 1974). This represents a very general life cycle of a temperate bumble bee colony. Both inter- and intra-specific variation make it difficult to specify precise environmental and social correlates associated with key changes within the life cycle.

Most social insects that live in enclosed nests are characterized by a homogeneous internal nest environment that can include near-constant temperature, humidity, and in many cases limited or complete lack of daylight exposure (Wilson 1971; Michener 1979; Seeley and Heinrich 1981; Weidenmüller et al 2002). Individuals within the nest are able to perform all tasks in the absence of light, and foragers are exposed to light only when performing tasks outside the colony (Tasei & Aupinel 1994). Thus, most experimental research on bumble bee colony development and social behavior has been done independently of variations in light and temperature (van der Blom 1986; Bloch and Hefetz 1999; Baer and Schmid-Hempel 2003; Sutcliffe and Plowright 1988). Furthermore, all patterns of bumble bee colony growth can be replicated under laboratory conditions, a fact that has been used to argue that colony development is independent of seasonal environmental changes (Alaux et al. 2005). However, there have been studies of

the effect of photoperiod and temperature on colony initiation in temperate species of bumble bees (Tasei 1994; Tasei et al. 1994; Beekman and Van Stratum 2000; Yoon *et al.* 2003). These studies have focused particularly on the effect of environment on gyne survival, rates of oviposition, and colony survival during the initiation phase when gynes are exposed to light while searching for a place to nest and during foraging to provision their first brood (Amin et al. 2007). One important finding from these studies in *Bombus terrestris* is an apparent correlation between the environmental conditions during gyne hibernation, photoperiod and temperature exposure of post diapause gynes, and variation in timing of the switch point from the growth phase to the reproduction phase of the colony (Beekman and Van Stratum 2000; Amin et al. 2007). Other factors such as number of workers, agonistic behavior, and workers' ovarian development apparently have no effect on this switch point (Duchateau 1991, Burns 2004; Alaux et al. 2005). However, these studies were not continued through the entire colony life cycle, nor have they been compared across other bumble bee species.

Seasonal photoperiod is an important cue for *Bombus impatiens* colonies to regulate growth and timing of production of reproductives (Hernandez in prep). In the current study I extend this investigation by using two methods to quantify daily patterns of activity at the colony level. Specifically, I tested the effects of different photoperiod regimes on changes of the daily patterns of worker movements to and from the nest, and I tested whether inactive behaviors of bees within the nest follow a circadian pattern and are dependent upon body size (an allometric relationship). I hypothesize that colonies will exhibit activity and rest patterns that reflect the photoperiod-induced circadian rhythms that occur in individuals. I also hypothesize that there are differences in the

patterns of behaviors such as foraging and rest among individuals of different size-classes within a colony. It is important to make the distinction between individual activity patterns and whole-colony activity patterns. Individual activity patterns give information about individuals' endogenous clocks and their effects on physiology and behavior. At the next higher level of organization, whole-colony activity patterns could reflect aggregate individual patterns that might or might not be synchronized but that give specific and unique rhythmic properties to the colony. For example, recent data from *B. terrestris* showed that colonies can be entrained to external photoperiod while individual castes exhibited differences in circadian behaviors (Yerushalmi et al. 2006; Stlezer et al. 2010). **In the current study I experimentally investigated the effects of photoperiod on the daily activity patterns of the bumble bee *Bombus impatiens* at both the colony and the individual level. At the colony level I investigated activity, and at the individual level I investigated resting behavior inside the nest. The experiments were conducted in laboratory conditions under controlled light-dark schedules of simulated natural and artificial periodicity. The combination of simulated natural and artificial photoperiods enabled dissection of key components of colony-level and individual-level responses to photoperiod.**

MATERIALS AND METHODS

Study Organism

B. impatiens colonies were commercially obtained from Koppert Biological Systems. Colonies consisted of one queen, a few workers (about 20 workers on average),

and brood at various stages of development (colony size type “C”). Each colony was transferred from the supplier’s transportation box to a wooden observation nest-box with a clear plastic top and removable cover that blocked light from entering the nest-box, thus simulating natural in-nest dark conditions. The nest-box was composed of a main chamber (25x25x15 cm) where the colony was placed, and a smaller chamber (8x25x15 cm) used for defecation and through which individuals passed between the brood area and the foraging area. The foraging area consisted of a mesh cage on a wooden frame (75x100x75 cm) that was connected to the nest box with a 25x3 cm transparent plastic tube (Figure 1a).

All colonies were fed fresh pollen supplied from local apiaries, and honey water solution provided by Koppert (methodology according to Plowright and Jay 1966, Cameron 1989, Cnaani et al. 2000). Pollen grains were ground and mixed with honey water solution to produce a homogeneous paste which was added directly to the colony box. Pollen and honey were added daily on a random time schedule to avoid synchronization of the colony to a feeding schedule. To avoid starvation or over-feeding, the amount of pollen given was normalized to the progression of brood growth (Evans et al. 2007). Honey water solution was provided once daily in the foraging area using 10 ml transparent plastic vials hung from the roof of the foraging area. Each vial contained two to four small holes at the base of the tube from which workers were able to extract the honey water solution. Daily volume of honey water was also sequentially increased to track the colony growth.

Experimental Design

Colonies were maintained in isolated rooms at the Animal Care Facility at the University of Missouri-St. Louis under constant temperature ($\pm 28^{\circ}\text{C}$) and humidity ($\pm 50\%$ relative humidity) conditions. The experiments were carried out in 2010.

Four different photoperiod treatments were tested using two colonies per treatment. Treatments were of two types (in hours of exposure, Light:Dark [L:D]): three constant L:D treatments (24h dark, 12:12, and 24h light), and a changing L:D photoperiod that simulated annual values of photoperiod similar to the St. Louis, Missouri latitude ($38^{\circ} 35' \text{N}$), at which the maximum total light is slightly less than 15 hours of light on the summer solstice. The initial simulated seasonal photoperiod day length was 12 h 45 min, which approximates the day length at the beginning of the social phase in the colony life cycle. Day length was increased by 15 minutes every fifth day until reaching a maximum of 15 hours of light, followed by a decrease in day length until the end of the experiment marked by the queen's death.

Foraging activity quantification

Directional events corresponding to an individual bee moving between the nest box and the foraging area were recorded using an electronic bee counter (Figure 1b). This electronic counter was similar to the bee activity recorder produced by Kevan et al. (2009) (See Hernandez and Garver in prep for a more detailed explanation of the counter design). Each photoperiod treatment had two colonies, each with an independent electronic bee counter that recorded events in bin sizes of 6 minutes for 24 hours a day starting on day 30 from the beginning of the experiment to the end of the colony cycle as

marked by the queen death (approximately day 110). Raw data were downloaded and processed using Microsoft Excel to produce complete colony activity files that were plotted using double-plotted actograms and analyzed for circadian parameters in MatLab (Mathworks 2007). Data captured by the counter were downloaded every three days in order to reset the counter and clean the foraging area access tube to avoid clogging that might lead to errors in the readings.

Individual rest patterns quantification

Serial digital photographs of both colonies for each photoperiod treatment were taken using a Nikon digital camera illuminated with two wireless mini slave flashes (Smith Victor PG250S) covered with red filters to reduce disturbance to the colony. Photographs were taken at 5 minutes intervals, as described in Eban-Rothschild and Bloch (2008), for approximately 48 hours beginning on day 50, which corresponds to the approximate midpoint of the colony life cycle and the peak of colony size. I counted the number of bees that did not move during 2 time intervals (10 minutes) every 30 minutes using the counter tool in Photoshop. The inter-tegular distance was measured as a proxy for body size (Goulson 2001, Peat et al. 2005, Couvillon et al. 2010,) as well as the substrate on which the bee was resting: on the wooden part of the box, on top of honey/pollen pots, or on brood cells.

Inactivity types recorded in this study were similar to the sleep-like stages in worker honey bees (Klein et al. 2008; Eban-Rothschild and Block 2008). Inactivity was determined when a bee did not move for a pair of photographs that correspond to 5 minutes intervals. Four types of relative immobility were recorded: (1) completely

immobile, abdomen or antennae raised above the substrate, (2) completely immobile, abdomen or antennae relaxed and touching the substrate, (3) minor changes in worker position such as small changes in the leg or antennae position or subtle changes in the abdomen, but never whole body position shifts even if the bee remained at the same location, and (4) workers incubating brood cells. In addition, I also scored spatial coordinates of each resting individual at all time points.

Statistical Analyses

I used Chi-square periodogram analysis to test for a significant rhythm in the activity recorded in the colony actograms of each photoperiod treatment. To detect phase of the colony circadian rhythm I used the ActogramJ plug-in for ImageJ (Schmid et al. 2011).

One way analysis of variance (ANOVA) with Tukey's post hoc multiple comparisons analysis was performed to compare the body size distributions of immobile bees among the different photoperiod treatments. When normality or homogeneity of variance assumptions were not met, I used the Kruskal-Wallis non-parametric Analysis of Variance test.

I tested for circular uniformity distributions of the time of day at which individuals remain motionless most frequently by means of the Rayleigh's test (Oriana ver 4.0, Kovach Computing Services, Anglesey, Wales, UK. 2010). Similarly, V tests for non-uniformity were used to test against the null hypothesis that density of the inactive bouts was randomly distributed along a 24 hour cycle (Batschelet, 1981).

Hartigan's dip test was used for assessing the body size distributions against unimodality using the package *dipTest* in R (R Development Core Team 2004). Survival analysis was used to test whether the duration of inactivity bouts was different between small and large workers in each photoperiod treatment (JMP 2001). Each worker inactivity bout was defined as an independent event, and the sum of all events per time unit (30 minute intervals) was the measurement used in the analysis. I did not score the same bee multiple times per measurement unit. However, the same bee could have been counted at different time points throughout the sampling. The goal of the experiment was to quantify the daily patterns of inactivity of colonies exposed at different photoperiods. In this sense, it is expected that bees show multiple inactivity events throughout the day. Spearman's correlation coefficient was used to test the degree of relationship between body size and duration of inactivity.

A Complete Spatial Randomness test was used to describe the spatial distribution of immobile bees in the colony box using a complete random distribution of the bees in the colony, with nearest neighbor distances as a null hypothesis (G measure and K measure, Kolmogorov-Smirnov test of CSR) in R (R Development Core Team 2004, package *spatstat*). Monte Carlo simulations were used to generate envelopes of confidence around the predicted random spatial distribution generated by the Ripley's K function (Ripley 1976, Bivand et al. 2008).

RESULTS

Colony-level activity patterns

There was a clear effect of photoperiod on the activity pattern during the entire social phase of the colony life cycle (Figure 2). All colonies exposed to light/dark treatments displayed a diurnal pattern of activity with a peak of activity during the first hours of the photophase (subjective day) and lowest activity during the initial hours of the scotophase (subjective night; Figure 2A, B). These colonies showed an increase in activity three to four hours before lights on, and the change from light to dark was marked by a decrease of activity three to five hours before this change (Figure 2A, B). Colonies exposed to the seasonal natural photoperiod treatment were entrained to the light-dark cycles. Colonies exposed to 12L:12D displayed robust circadian rhythms corresponding to the light-dark schedule (mean period = 24.0h). Colonies in constant dark conditions exhibited a robust free-running rhythm with a mean period of approximately 24.2h \pm 0.1h (Figure 2C, Figure 3). Colonies maintained in constant light showed a weak rhythm with a mean period of 23.8 \pm 0.2 h (Figure 2D). In all photoperiod regimes, activity was particularly strong during the beginning of the colony life cycle and weakened as the colony aged (Figure 3), with a loss of rhythmicity toward the end of the colony life cycle.

During the colony life cycle, daily patterns of activity showed variation in terms of the time of day for the highest peak of worker activity, where the activity patterns in any day were slightly different from the activity patterns of the next day. There was a

tight matching of directional events of individuals leaving or entering the colony (Figure 4).

Daily patterns of rest-like behaviors

In all photoperiod regimes, worker rest cycles coincided with the exposed experimental light-dark schedules (Figure 5). The photoperiod treatments that included lights on/off schedules showed more bees motionless during the scotophase (subjective night) than during the photophase (subjective day) (Figure 6, table 1). In these treatments the proportion of inactive individuals was non-uniformly distributed with higher density of inactivity bouts toward the middle of the subjective night. In contrast, the mean number of individuals resting in the colonies exposed to 24 hours of constant dark or constant light was equally distributed throughout the 24 hour cycle (Figure 6 table 1).

Colonies exposed to a seasonal photoperiod treatment produced a larger number of workers than in other tested photoperiod treatments (Hernandez et al. in prep). For this reason, all comparisons between treatments were normalized to the number of adult individuals in the colony at the corresponding time point. Subsequent analysis showed a significantly lower mean percentage of the population exhibiting inactive bouts in colonies exposed at 12L:12D with all other treatments not significantly different from each other (ANOVA $F=5.05$, $p=0.0027$, post hoc Tukey's multiple comparison test, Table 2). On the other hand, there were non-significant differences between photoperiod treatments at the highest ($22.5 \pm 6.22\%$; mean \pm SD) and lowest ($3.14 \pm 0.12\%$; mean \pm SD) peaks in the mean proportion of inactive workers (Table 2). The number of inactive

events reported for the seasonal photoperiod treatment was more than two times larger than for any other treatment (Figure 7).

Worker inactivity bouts were mostly of short duration regardless of the photoperiod treatment applied. Over 55% of the inactivity events lasted less than 10 minutes. Bouts lasting longer than 30 min constituted less than 10% of worker inactivity events (Figure 8). The longest resting bout was in a large worker (6.52 mm – above 90th percentile) of the seasonal photoperiod treatment and lasted 110 minutes. In treatments with light-dark cycle schedules the longest individual resting bouts occurred during the scotophase, but there was no significant difference in the mean duration of resting bouts during photophase and scotophase in either the seasonal photoperiod treatment (Wilcoxon test 17.37 ± 1.32 (mean \pm SE, lights off); 15.10 ± 0.83 (mean \pm SE, lights on), $X^2=0.187$) or the 12L:12D treatment (Wilcoxon test 19.93 ± 2.42 (mean \pm SE, lights off); 13.55 ± 1.53 (mean \pm SE, lights on), $X^2=0.187$).

Interaction of photoperiod and body size

Colonies exposed to the seasonal and the 12L:12D photoperiod regimes showed the smallest overall mean body size (ANOVA $F=3.305$, $p=0.021$ post hoc Tukey's multiple comparison test; Table 3). The 12L:12D and 24L photoperiod treatments had the smallest mean body size of inactive workers (ANOVA $F=66.57$, $p<0.0001$ post hoc Tukey's multiple comparison test). There was no significant difference in body size between inactive workers and whole-colony body size distributions in the 12L:12D and 24L photoperiod treatments. In contrast, the mean body size of resting workers was larger

than the whole-colony body size in both the seasonal and 24 DD photoperiod treatments (Table 3).

The seasonal photoperiod and the 12L:12D treatments colonies showed a bimodal distribution of body sizes with a larger mode that represented smaller bees (seasonal treatment mode = 4.5 ± 0.6 mm, 12L:12D mode = 3.85 ± 0.5 mm, mean \pm SE) and a second, smaller, mode that represented large bees (seasonal treatment mode = 6.5 ± 0.44 mm, 12L:12D mode = 5.2 ± 0.4 , mean \pm SE) (Hartigan's dip test $p < 0.05$ for both treatments; Figure 9). In contrast, during constant light and dark treatments the whole-colony body size was normally distributed (Hartigan's dip test $p > 0.2$; Shapiro-Wilk test for normality (**24L**; 4.52 ± 0.66 (mean \pm SE); $W = 0.969$ $p = 0.560$; and **24D**; 4.49 ± 0.43 (mean \pm SE); $W = 0.932$ $p = 0.1237$; Figure 9), although in both cases sample sizes were small (24L $n = 28$; 24D $n = 25$).

Using ranked body size groups of the seasonal and the 12L:12D photoperiod treatments, I tested for differences in the body size of inactive bees between the subjective day versus the subjective night. There is significant variation in body size of inactive workers in the seasonal photoperiod treatment, with an average larger body size of inactive workers occurring during the photophase period of the experiment (5.1 ± 0.07 vs 4.7 ± 0.06 (mean \pm SE), Wilcoxon test $X^2 = 7.5046$ $P = 0.0062$). When the data were further partitioned between large and small individuals, small worker inactivity appears to cycle with a peak during the subjective night whereas there was no apparent cycle of inactivity for the larger workers (Figure 10). In contrast, in the 12L:12D photoperiod treatment, there is no significant variation of body size between the subjective night

(scotophase) and the subjective day (photophase) (4.13 ± 0.07 vs 4.11 ± 0.06 (mean \pm SE) Wilcoxon test $X^2=2.9212$ $P=0.087$).

I found a positive relationship between duration of inactivity bouts and body size in the seasonal photoperiod treatment wherein large bees tend to remain inactive for longer periods of time than smaller bees (14.11 ± 0.69 vs 24.05 ± 2.4 minutes (mean \pm SE; survival analysis Wilcoxon test $X^2=27.66$ $P<0.0001$; Figure 11). In contrast, in all remaining photoperiod treatments there was no relationship between body size and time spent motionless (Table 4).

Effect of photoperiod on sleep-like behaviors

Both large and small workers exhibit equal proportions of all of the three stages of sleep-like behavior, excluding incubation, independently of the photoperiod treatment. There were no body size or bout duration differences between the photoperiod treatments with respect to the sleep-stage behavior observed in all treatments. In all treatments workers displayed incubation-like bouts on average no more than 5 minutes which were on average smaller than those of bees displaying other types of rest-like behaviors (Figure 12). These inactive incubating workers were almost exclusively restricted to the center of the brood area.

Effect of photoperiod on queen inactivity

The queen had frequent but short inactivity periods in all photoperiod treatments. More than 75% of the queen's inactivity bouts were of 5 minutes duration with no bouts above 20 minutes duration. These inactivity bouts were equally distributed in all

photoperiods (Figure 13). In all treatments the queen had high constancy for particular areas inside the brood section of the nest where she would spend her inactive time. These resting areas were particularly close to egg clusters or larval clusters at their initial stages of development.

Spatial distribution of motionless workers

In all photoperiod treatments evaluated, I found higher densities of inactive small workers than inactive large workers corresponding to the worker size ratio of 1:4 that is normally found in the colonies (Average intensity 1.54 vs 0.38 bees per square cm).

In the seasonal photoperiod and 12L:12D photoperiod treatments, resting workers were distributed significantly differently from a Poisson distribution, clustering more than expected under complete spatial randomness (Kolmogorov-Smirnov test of CSR $p < 0.0001$, Figure 14). In the seasonal photoperiod treatment inactive workers were found farther from the center of the brood than in the other treatments (ANOVA $F = 90.01$ $p < 0.0001$ post hoc Tukey's multiple comparisons test). In the seasonal and 12L:12D photoperiod treatments neither body size rank nor lights on/off had an effect on the spatial distribution of inactive workers during the 24 hour cycle. However, there was a clear spatial segregation of inactive individuals based on body size in the seasonal photoperiod treatment where large and small workers were more likely to be found inactive toward the periphery away from the center of the brood area. However, large bees were more likely to be found farther away from the center of the brood area than small bees (Kruskal-Wallis test $X^2 = 38.714$ $P < 0.0001$, Figure 15). In contrast, in the constant photoperiod treatments (12L:12D; 24L; and 24D) most inactive bees were found

within the brood area. In all treatments, workers that remained in the center areas with the brood or near pollen or nectar sources were more likely to be small individuals.

DISCUSSION

Exposing *B. impatiens* colonies to different photoperiod regimes had a significant effect on the circadian clock with respect to colony-level foraging and sleep-like patterns of activity. The colony-level circadian clock for this species showed characteristics similar to an individual circadian clock: 1. colonies exhibited free-running activity rhythms under constant DD conditions, and 2. colonies were entrained to the light-dark cycle to which they were exposed. Under constant LL conditions, colonies showed a weak rhythm with short resting periods. This weak rhythm could be the result of a loss of circadian synchronization among individual worker clocks (probably foragers) as suggested by Stelzer et al. (2010) for *B. terrestris*. At the same time there could be circadian entrainment agents inside the nest box to maintain a relative stable cycle under constant DD conditions. One possible candidate for such an entrainment agent is the queen, as demonstrated in honey bees. For example, when honey bee queens are introduced into colonies entrained to different circadian rhythms, those colonies synchronize their rhythms to those of the newly introduced queens but not to those of introduced workers (Moritz and Sakofski 1991; Moritz 1994). Or, as suggested by Stelzer et al. (2010), external cues such as temperature fluctuations, light intensity, or even resource cycles (nectar and pollen) could entrain foragers to cycle. However, these variables were controlled in this study, rendering them unlikely to be sources of entrainment.

Taken together, the results reported in this study support the notion of a colony circadian clock found in other social insects such as honey bees (Moritz and Fuchs 1998; Toma et al 2000; Bloch 2010), bumble bees (Bloch 2010; Stelzer et al 2010), stingless bees (Bellusci and Marques 2001), and ants (Lone and Sharma 2011). In particular, the circadian periods observed for all photoperiod treatments were most closely comparable to those found in *B. terrestris* (Stelzer et al. 2010). In addition to confirming, via independent study, the circadian activities of temperate zone *Bombus* as previously reported for *B. terrestris*, the present study is among the first to include daily activity patterns encompassing the social phase (from the production of the first workers to the queen's death) of a social insect life cycle.

The tight correspondence between the daily in and out movement of individual workers from the nest to the foraging area indicates that foragers engage in quick foraging trips and soon return to the nest. However, I observed a number of workers that would not go to the foraging vials but instead would walk on the foraging area, possibly scouting. Under natural conditions, foraging trips are likely to last longer than in the controlled colonies, therefore the traffic dynamics could differ from my results. Preliminary data indicate, however, that similar traffic patterns are observed in field colonies for this species, the only difference observed thus far being the duration of each foraging trip (Hernandez in prep).

In a previous study, I found a significant effect of photoperiod on population size throughout the colony life cycle (Hernandez et al in prep). In the present study the actogram data for each photoperiod treatment showed foraging intensity to be correlated with population size, with lower levels of activity occurring at lower population sizes (the

tail end of the actograms). In all treatments, I found a decline of activity toward the senescence of the colony and a coinciding loss of rhythmicity. This decline of activity is also correlated with a reduction in the queen's oviposition rates and an increase of worker's male production (Wilson 1971; Cameron 1989; Bloch 1999). Only the constant DD photoperiod showed an increase of activity toward this last stage of the colony life cycle, similar to the "death dance" described in *B terrestris* workers (Stelzer et al. 2010) and in *Drosophila* (Levine et al. 2002a).

Colonies exposed to the seasonal photoperiod treatment adjusted their activity to the changing day/night lengths of the light cycle. It is important to note the mean circadian period (24.1h) is an average of the daily circadian periods throughout the colony life cycle. Because the day length was adjusted only at the change between lights on to lights off, the actograms show that, at least during the first half of the colony life cycle, the period of activity changed in a manner corresponding to the stepwise increase of day length and in a weaker correspondence to the subsequent decrease. Similar changes in circadian periods of activity have been reported for honey bee colonies (Bloch et al. 2006).

In the simulated natural photoperiod and the 12L:12D photoperiod regimes, there was an increase in the workers' activity patterns in the hours before the beginning of the photophase (subjective day). Peak activity levels occur during the subjective day, and, as seen in other diurnal organisms, these peaks occurred more frequently at the initial hours of the photophase (Aschoff 1979; Levine 2002a). It was not possible to determine if the increase in the morning activity was due to bees leaving the colony to forage or an increase in other behaviors, such as defecation, that required leaving the nest box. In *B.*

impatiens, I did not observe an increase in the activity levels at the end of the photophase similar to those described for colonies and workers of *B. terrestris* (Stelzer et al. 2010) and solitary species such as *Drosophila* (Rosbash et al. 2003). In contrast, colonies exhibited a rapid decrease in activity a few hours before lights off at the end of the subjective day. This result suggests that colonies anticipated the change from light to dark by reducing activity between the colony and the foraging area before the beginning of the scotophase. One probable explanation is that although food was provided randomly throughout the 24 hour cycle, it was also normalized to colony size and was not provided *ad libitum*. Thus, the time foragers spent in consuming the resource was reduced to a few hours after the feeding time (or the beginning of the day on days when food was provided during the subjective night); therefore, there was little reward in new foraging flights near the end of the day. This result is similar to that found in field colonies of *B. terrestris* and *B. pascuorum* (Stelzer and Chittka 2010) but is in contrast to colonies of *B. terrestris* kept under laboratory conditions (Stelzer et al. 2010).

Colonies exposed to longer nights exhibited more individuals sleeping. The proportion of inactive individuals from the total population size was independent of photoperiod and population size. This result suggests that the colonies always maintain a subset of “reserve” individuals in relative proportion to the colony size (Jandt and Dornhaus 2009; Jandt and Dornhaus 2011). In small populations the ratio of incubating to inactive individuals is higher than in large colonies. This increased incubation proportion may be associated with lowered internal temperatures in small colonies where the need for incubation could be greater, although this hypothesized relationship requires focused examination. Jandt (2010) showed that inside the colony, larger bees have larger home

ranges and that they are normally located farther from the center of the colony and the queen. Large workers are more likely to be foragers with stronger circadian rhythms than small workers, which tend to become in-nest workers (Yerushalmi et al. 2006).

Therefore, it was surprising to find that the small body size class showed a stronger inactivity rhythm than the large body size class, which has relatively equal proportions of inactive individuals at any time point during the 24 hour cycle. In honey bees, foragers sleep at all times even during the day when not foraging, and there seems to be a very organized social sleep schedule that depends on individual foraging schedules, while at the same time maintaining a higher proportion of foragers sleeping at night (Klein et al. 2008, Klein and Seeley 2011). I found a less organized social sleep in *B. impatiens*; although a significantly higher proportion of bees slept at night, the proportion of foragers sleeping was not significantly higher at any point during the 24 hour cycle independent of the photoperiod treatment. This result may be an artifact of the experimental design due to an absence of the long foraging flights as found in natural conditions perhaps leading to more time that large bees spend in the nest throughout the 24 hour cycle.

Similar to honey bees in which older bees, typically foragers and food storers, will have longer uninterrupted bouts of inactivity (Klein et al. 2008), I observed large bees exhibiting longer bouts of inactivity than smaller bees. Inactivity bouts in *B. impatiens* are longer than those reported for honey bee foragers (about 5 minute bouts) and nurses (less than 3 minutes) (Klein et al. 2008). Inactive behavior of the queen resembles that of small workers, with short bouts of activity and a spatial preference for inactivity bouts in the brood section of the nest box. When brood is present, *B. terrestris*

queens and in-nest workers exhibit arrhythmic patterns of locomotor activity attending the brood around the clock Bloch (2011). These results from *Bombus* are consistent with those found in mated honey bee queens, in which there was no daily rhythmic synchronization of behaviors regardless of season, photoperiod, or temperature (Johnson et al. 2010).

There was a clear effect of photoperiod and body size on the spatial location of inactive bees. In large colonies, where traffic within the brood area is high, inactive individuals tend to be located toward the periphery of the brood. Large workers were more likely to be clustered farther away than smaller bees. These results suggest that small individuals that remain close to the brood area minimize the time spent moving from their inactive sites to the brood. However, small colonies that are subject to constant light or dark regimes will have all the worker force sleeping within the brood area, possibly helping to maintain a constant temperature. Evidence suggests that there is a strong correlation between social insects' group size and division of labor with a strong adaptive value (Bonner 2004, Dornhaus et al. 2009, Holbrook et al. 2011). I found that smaller colonies have lower size dimorphism than larger colonies, which in turn is associated with changes in the diurnal patterns of activity and probably division of labor within a colony.

CONCLUSIONS

Environment has been a major selective force in the evolutionary trajectory of species. In social insects, understanding such effects proves challenging due to the behavioral complexity and plasticity associated with social , which adds a layer of

complexity in potential responses to selective factors acting above the individual level. How social colonies respond to environmental pressures in terms of changes in the social structure and division of labor should be a priority in our understanding of the evolution of social behavior. My results provide initial evidence on the intra-colonial regulation of the seasonal life cycle on temperate *Bombus* and the colony response to photoperiodic changes. These results also extend our understanding of size-based differences in the expression of circadian rhythmicity and its effects on the internal regulation and spatial distribution of individuals within the colony. In this context, an important question to be addressed is the effect of photoperiod on the evolution of social systems. Photoperiod is strongly associated with seasonal changes in temperature and food availability, thereby imposing a strong selective force for species to predict changes in environment by using such reliable cues. My findings indicate that colonies exhibit constant assessment of photoperiod and, via differences in individual activity patterns, translate this information into fine-tuned internal social conditions and developmental processes.

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Table 1. Circular statistical analysis for inactive workers of *B. impatiens* at different photoperiod treatments. *P* values in bold represent significance < 0.05.

Photoperiod Treatment	Mu (mean vector)	Z value (sample size)	Raleigh Test
Seasonal	20:47 PM	26.96 (415)	<0.001
12L:12D	17:32 PM	3.425 (142)	0.033
24D	9:30 AM	2.303 (103)	0.1
24L	3:26 AM	0.695 (70)	0.499

Table 2. Percentage of inactivity bouts in colonies of *B. impatiens* exposed to different photoperiod regimes. Average values in bold represent significance < 0.05.

Photoperiod Treatment	Percentage Peak inactivity	Percentage Lowest value inactivity	Average percentage inactivity (Mean +/- (SE))
Seasonal	20.76%	3.07%	12.27(1.09)%
12L:12D	16.92%	3.07%	8.46 (1.09)%
24D	20.12%	3.33%	14.26 (1.11)%
24L	31.25%	3.12%	10.45 (1.09)%

Table 3. Differences in distribution of whole colony body size and inactive individuals in *B. impatiens*. *P* values in bold represent significance < 0.05.

Photoperiod Treatment	Mean whole-colony body size	Mean inactive individuals body size	p-value
Seasonal	4.26 ± 0.07	4.98 ± 0.04	0.0001
12L:12D	4.25 ± 0.10	4.23 ± 1.03	0.907
24D	4.71 ± 0.16	5.20 ± 0.07	0.0001
24L	4.63 ± 0.15	4.59 ± 0.12	0.8850

Table 4. Correlations between worker body size and duration of inactive bouts in the bumble bee *B. impatiens*. *P* values in bold represent significance < 0.05.

Photoperiod Treatment	R ²	p-value
Seasonal	0.4410	0.0036
12L:12D	0.054	0.5363
24D	-0.026	0.4045
24L	-0.05	0.446

FIGURE LEGENDS

Figure 1. A. Experimental set up used to investigate the effects of photoperiod on colony development in the bumble bee *Bombus impatiens*. B. Bee activity counter used to measure colony activity patterns in the bumble bee *B. impatiens*.

Figure 2. Daily activity patterns under different photoperiod treatments of colonies of *B. impatiens*. There is a clear increase in the overall activity during the photophase in colonies exposed to treatments with lights on/off schedules (panels A, B), whereas colonies exposed to constant DD or LL conditions showed a weaker circadian rhythm of activity (panels C, D). Each bar represents an hour of the day, and the height of the bars represent the mean activity normalized by population size. Dark bars represent the subjective night (scotophase), and white bars represent the subjective day (photophase).

Figure 3. Double plotted actograms of foraging activity under different photoperiod treatments of colonies of *B. impatiens*. Colonies adjusted their activity patterns in response to the photoperiod treatment. The red lines represent the light/dark schedule throughout the colony life cycle. All mean periods reported are significantly different from a random period at p values < 0.001

Figure 4. Daily patterns of activity for a subset of seven days (days 40 to 47 from the beginning of the experiment) under the 12L:12D photoperiod treatment for a

colony of *B. impatiens*. There was a tight correspondence of activities patterns among workers moving in and out of the nest box. Red lines represent individuals moving from the box to the colony area, and blue lines represent individuals moving from the foraging area to the colony box. Black bars in the x axis represent the subjective night (scotophase).

Figure 5. Daily patterns of rest-like behaviors of *B. impatiens* colonies under different photoperiod treatments. Colonies allocate a higher proportion of workers to rest during the scotophase hours in colonies with a lights on/off photoperiod schedule. Colonies with constant DD or LL photoperiod schedules show no circadian patterns of rest-like behaviors. Each point represent one hour. Shaded areas represent the subjective night (scotophase), white areas represent the subjective day (photophase). Data are taken at day 50 from the beginning of the experiment, which corresponds to the largest colony size and an average mid-point for the colony life cycle

Figure 6. Rose diagrams of the rest-like behaviors of *B. impatiens* under different photoperiod treatments. There is a significant increase in the proportion of resting workers during the scotophase than during the photophase in colonies under a seasonal or a 12D:12L photoperiod schedule, with no effect in colonies exposed to a constant DD or LL photoperiod. Each bar represent one hour. The red arrow shows the mean vector of inactivity calculated for each colony. The red circle

represents the 95% confidence interval obtained by Rayleigh's test. A significant value was obtained when the mean vector (red arrow) was larger than its radius.

Figure 7. Percentage of inactive workers of *B. impatiens* under different photoperiod treatments. The seasonal photoperiod treatment shows a clear significantly higher proportion of inactive workers than any other treatment (ANOVA $p > 0.001$ $n = 790$ events (8 colonies))

Figure 8. Duration of inactive bouts in terms of number of individuals in *B. impatiens*. Blue line represent a smoothed function of the distribution ($n = 8$ colonies).

Figure 9. Whole-colony body size distributions of *B. impatiens* under different photoperiod treatments ($n = 8$ colonies). Inter-tegular distance was used as a proxy for body size (Goulston 2001).

Figure 10. Comparison of the daily patterns of rest-like behaviors in the seasonal photoperiod treatment between small workers (top, in red) and large individuals (bottom, blue). There is an apparent circadian pattern of resting behavior for the small body size workers, whereas there is no apparent cycle for the large workers. Grey areas correspond to the subjective night (scotophase).

Figure 11. Relationship between duration of sleep-like intervals and body size in the seasonal photoperiod treatment in *B. impatiens*. The size and color of the circles

represent abundance (number of individuals). There is a positive relationship, with larger individuals exhibiting longer rest-like bouts (Pearson correlation: $p < 0.0036$; $R^2=0.44$).

Figure 12. Sleep-like behaviors in the bumble bee *B. impatiens*. A. Incubation B.

Workers completely immobile, abdomen or antennae raised above the substrate. C

Worker completely immobile, abdomen or antennae relaxed and touching the substrate

Figure 13. Frequency of the queen inactive bouts as an effect of photoperiod. Each color represents a time interval. In all photoperiod treatments, queens exhibited short inactive bouts in more than 70% of all events.

Figure 14. Examples of spatial point pattern distributions of inactive behaviors in colonies of *B. impatiens* under different photoperiod treatments. Yellow Circles represent the brood area, and black cylinders represent the box entrance.

Figure 15. Spatial distribution of inactive small workers (left) versus inactive large workers (right) obtained from a colony exposed to a seasonal photoperiod treatment where peaks in the landscape represent a higher density of inactive individuals. Both large and small workers tend to cluster toward the periphery of the nest; however, large individuals are on average farther from the center of the

colony than small individuals. Yellow circles represent the brood area, and black cylinders represent the box entrance.

A

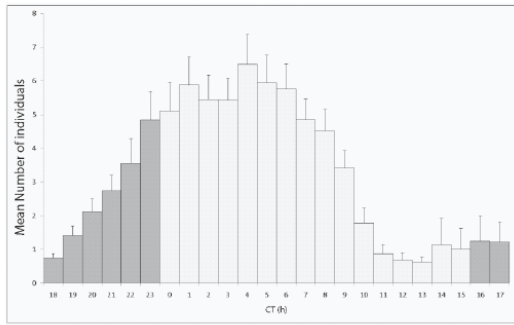


B

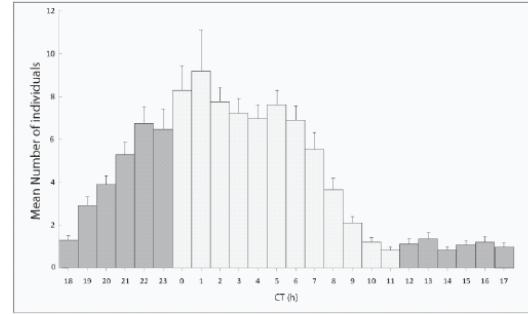


Figure 1

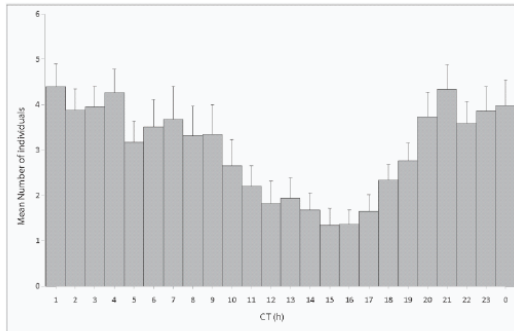
A Seasonal



B 12:12



C DD



D LL

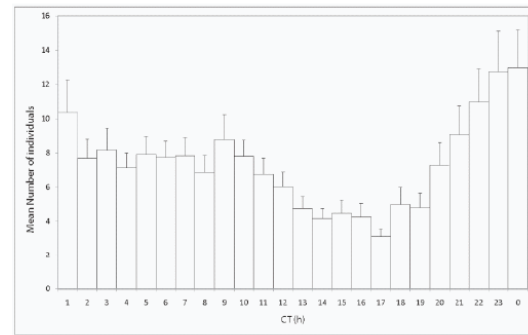


Figure 2

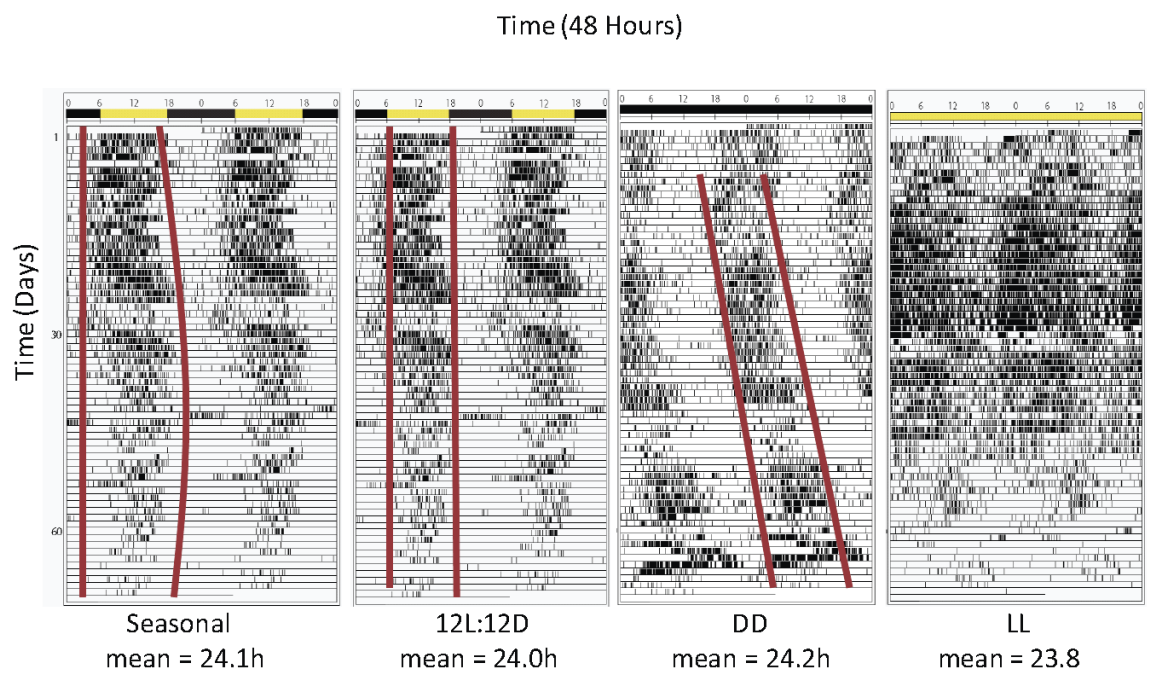


Figure 3

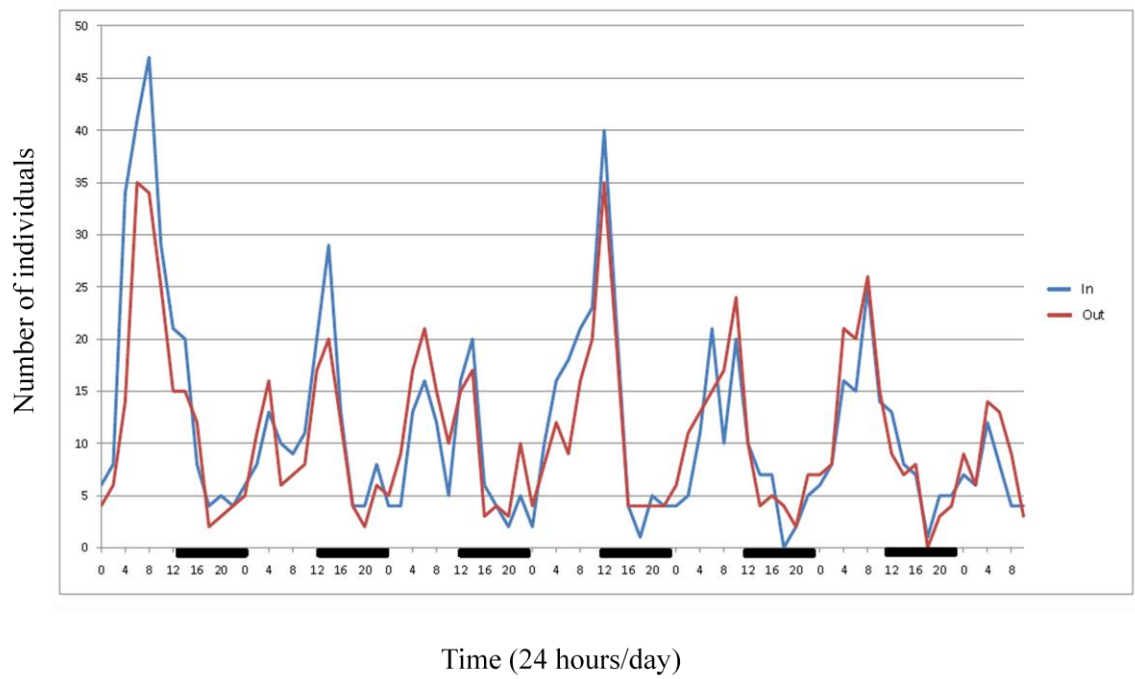
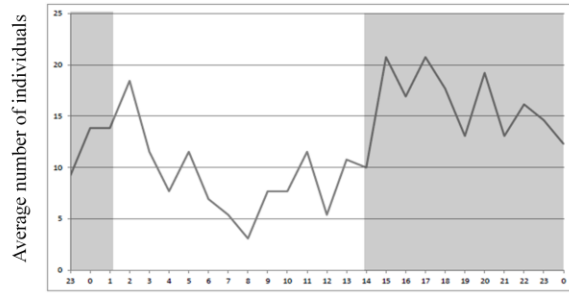
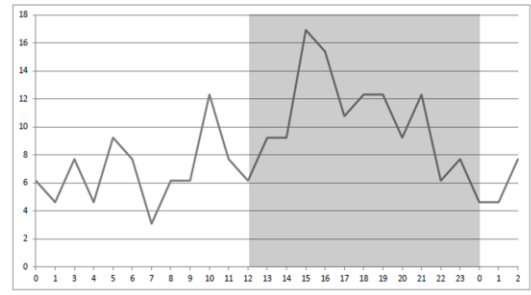


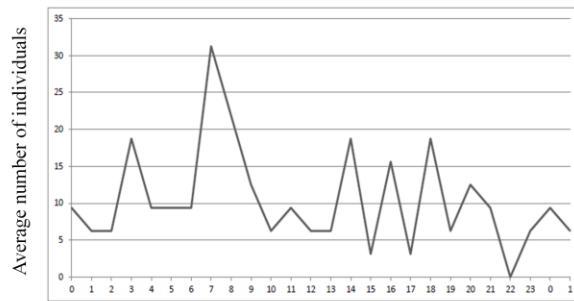
Figure 4



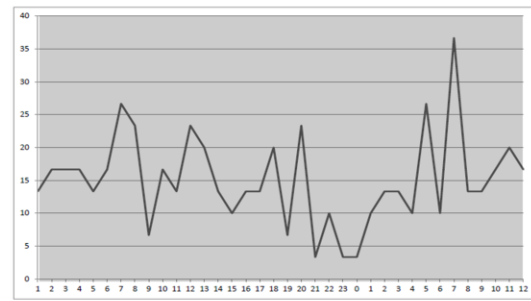
Seasonal



12L:12D

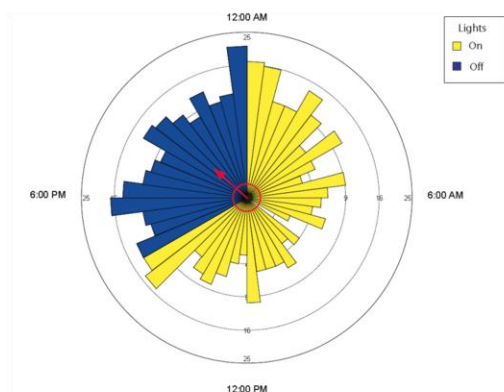


DD

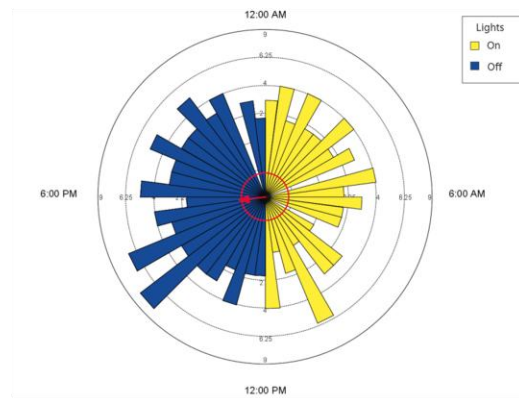


LL

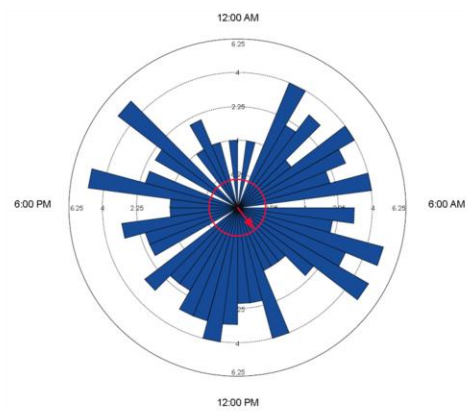
Figure 5



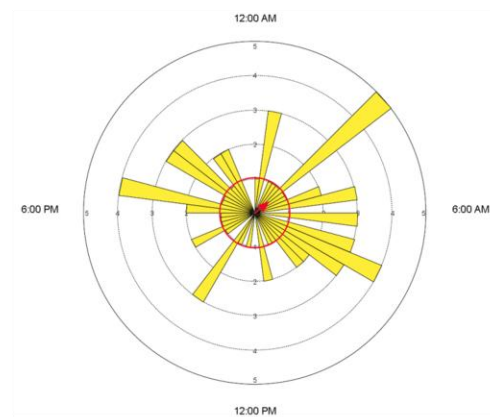
Seasonal



12L:12D



DD



LL

Figure 6

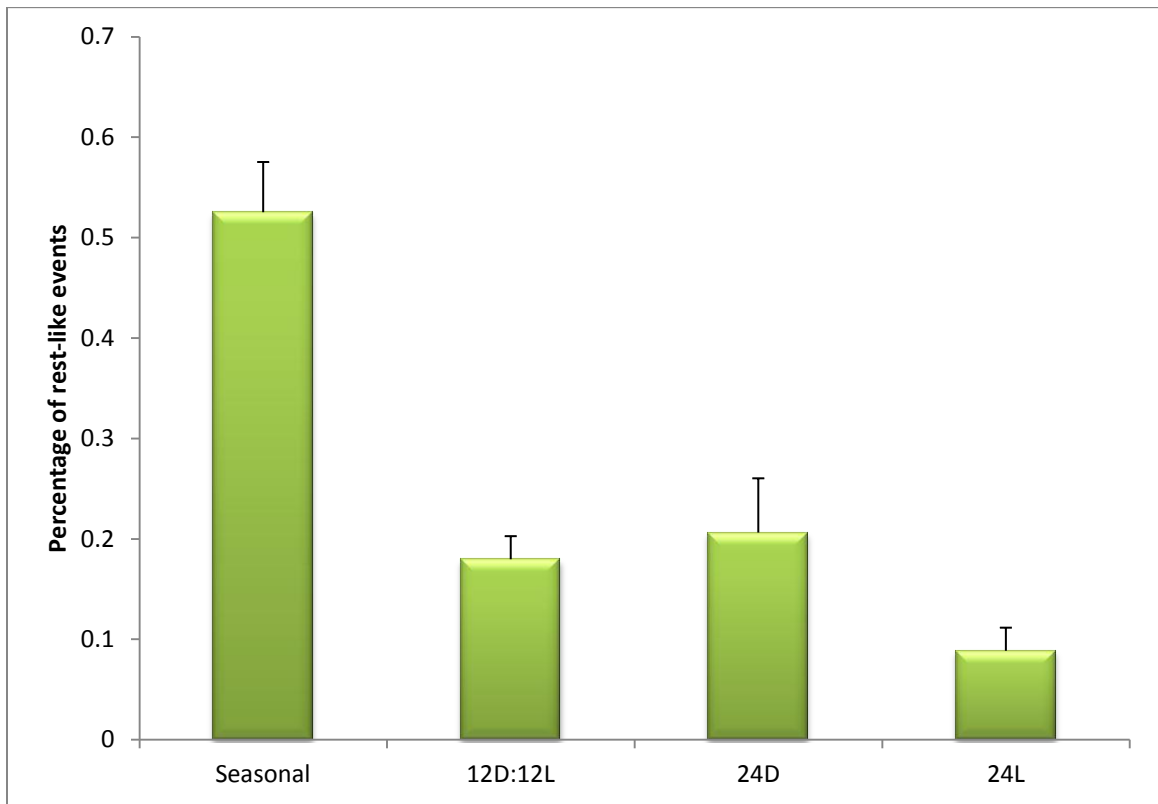


Figure 7

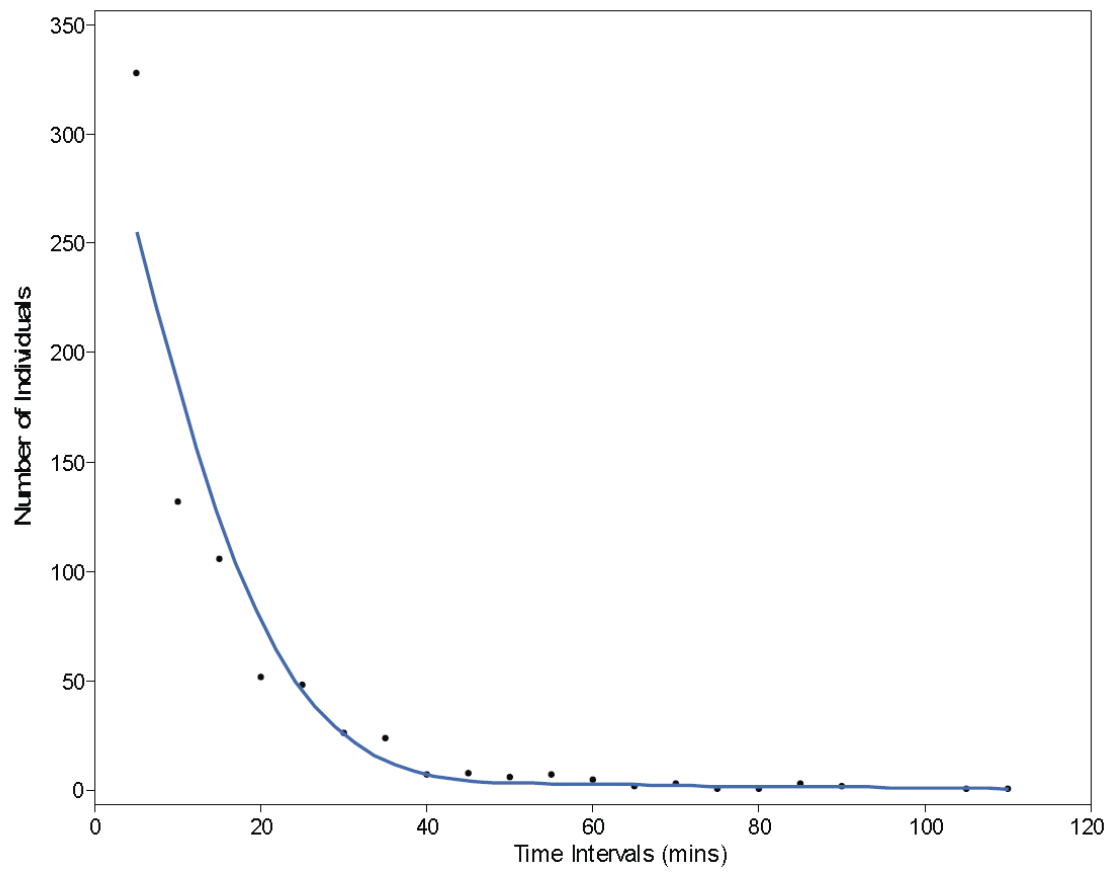


Figure 8

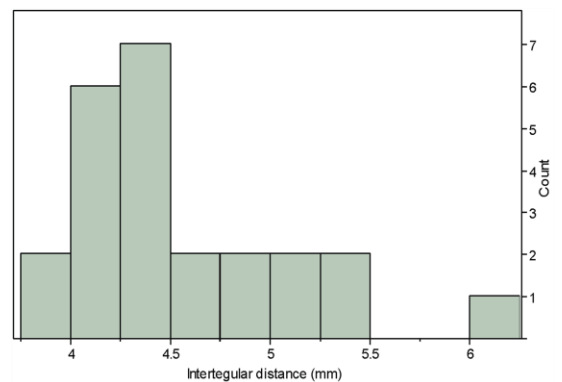
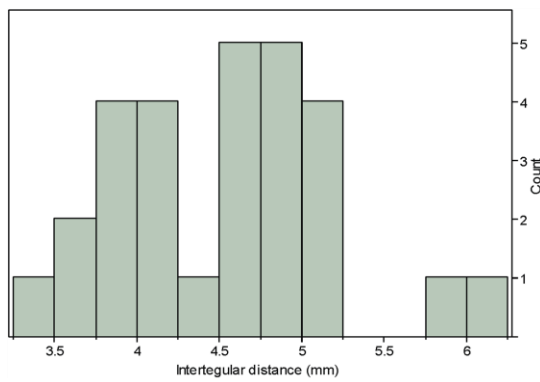
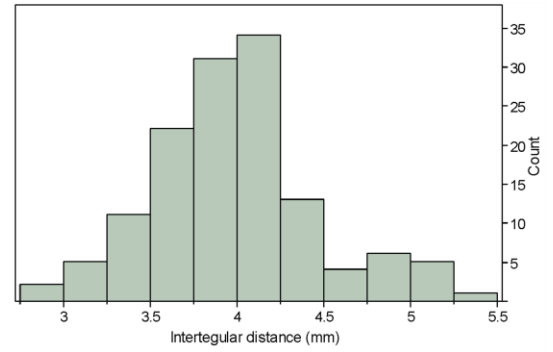
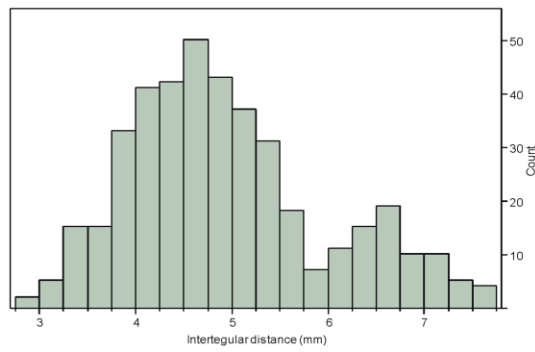


Figure 9

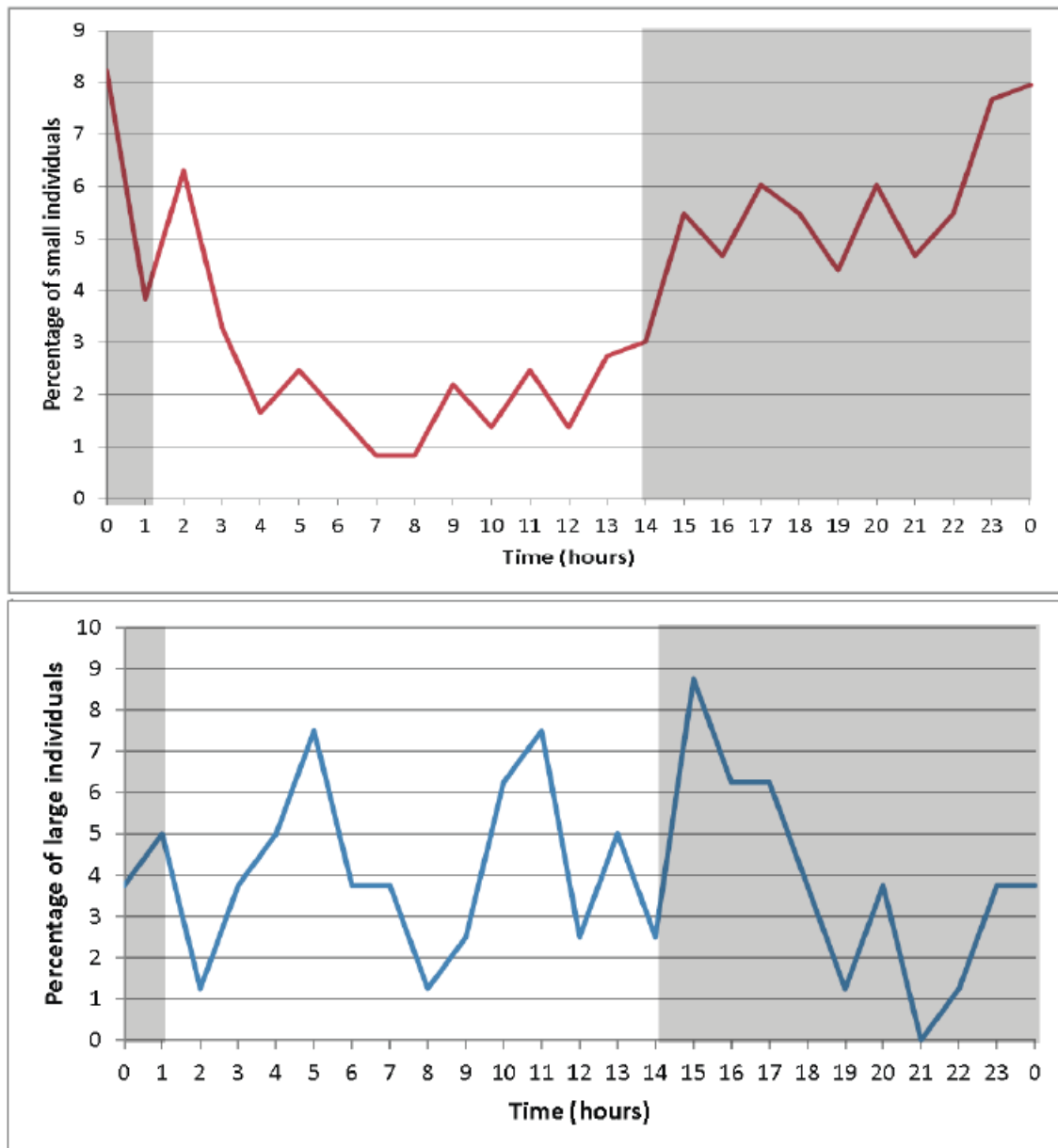


Figure 10

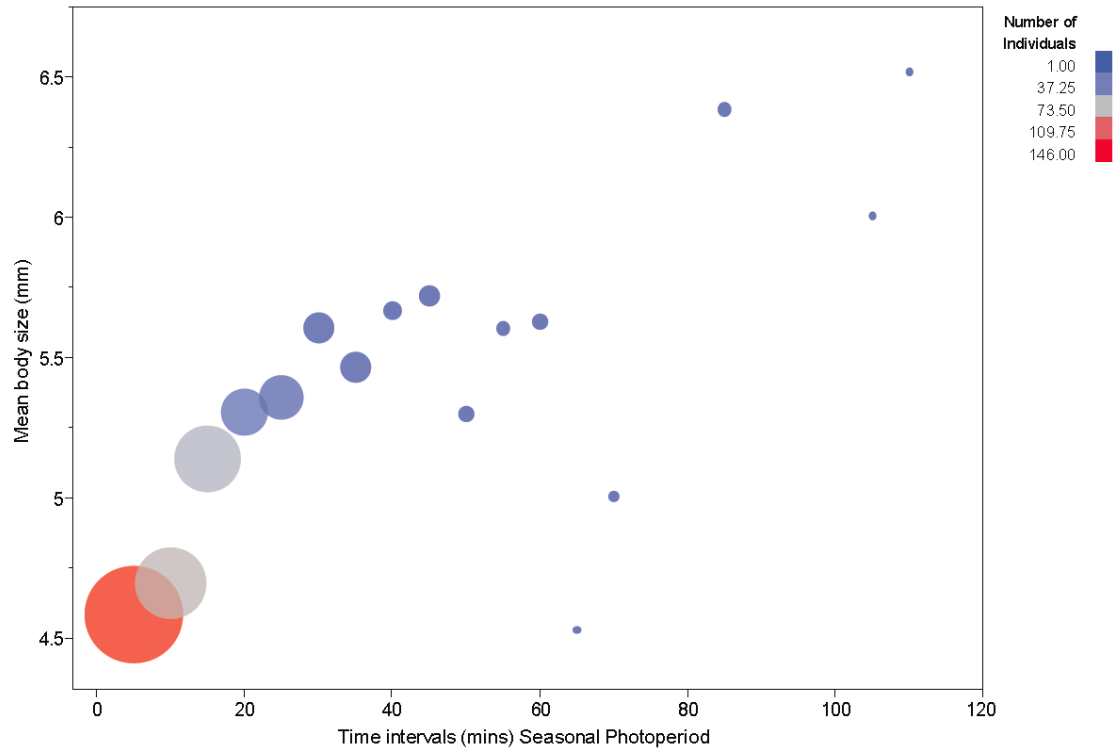


Figure 11



Figure 12

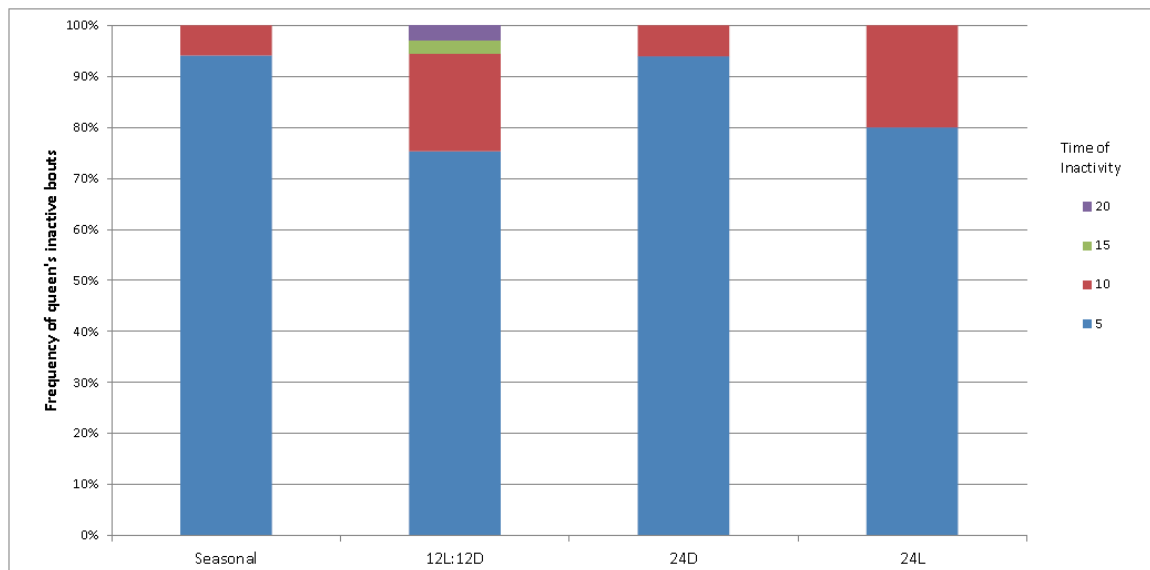


Figure 13

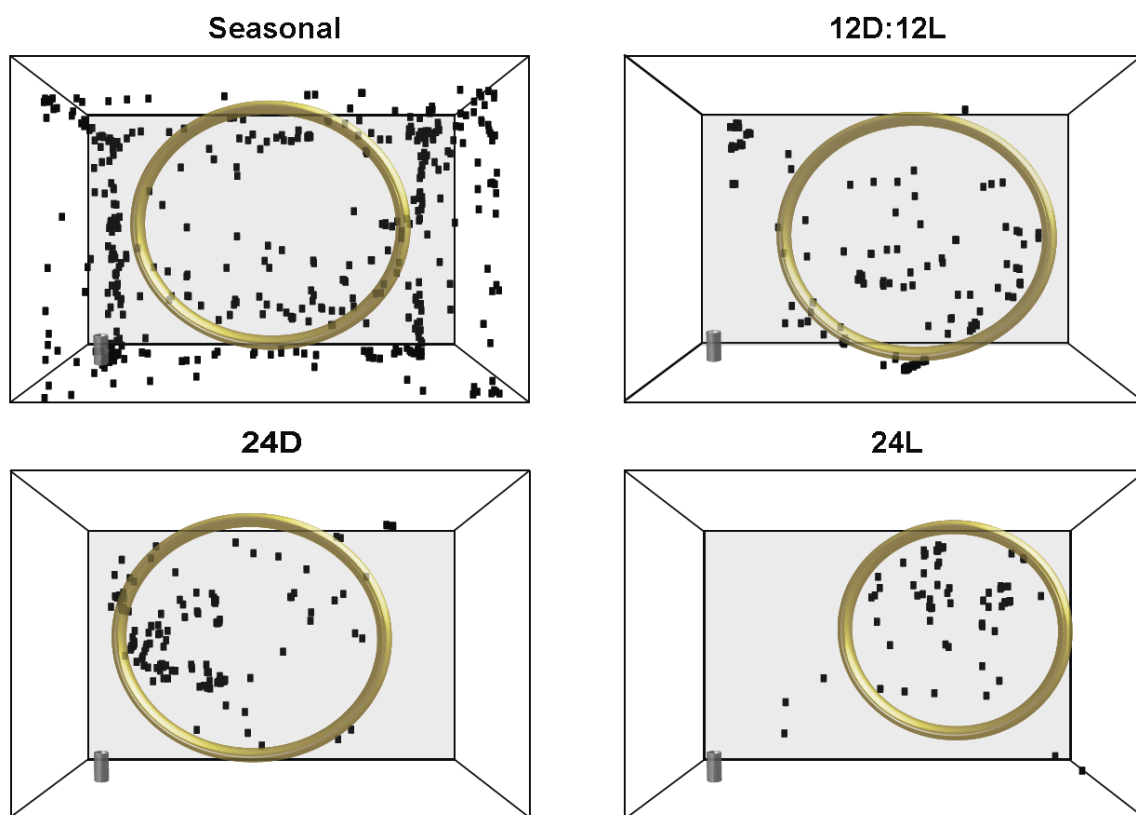


Figure 14

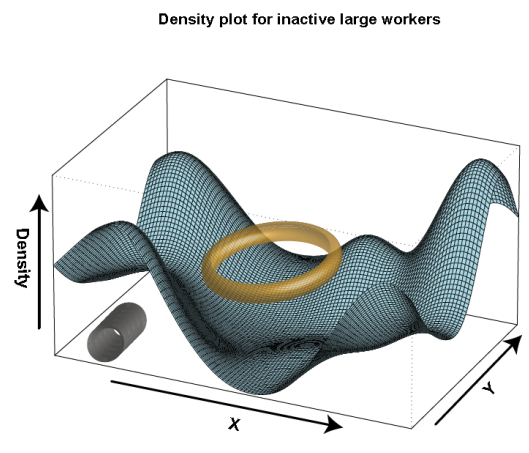
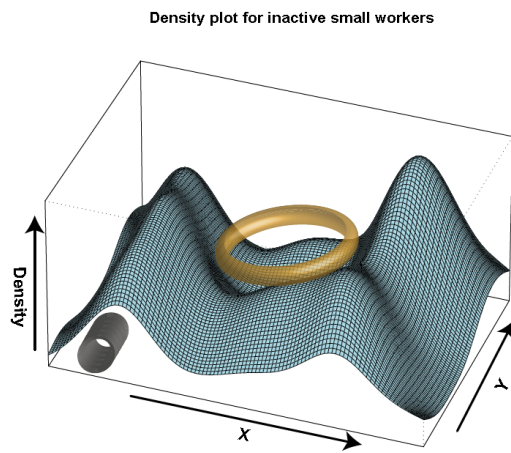


Figure 15

**CHAPTER 3 Behavior and body size of the bumble bee *Bombus impatiens* L.
(Hymenoptera: Apidae) under different photoperiod regimes.**

Abstract

In this set of experiments I evaluated individual behaviors in colonies of the bumble *Bombus impatiens* over the social phase of the colony's life cycle that were exposed to three photoperiod regimes. I measured the change in the proportion of the frequency of four main functional tasks; brood care, non-social behavior, guarding and foraging of colonies along the social phase of the colonies life cycle. I found that colonies maintain a constant proportion of functional tasks that is independent of photoperiod and colony age. Then I explored how individual behaviors differed in colonies under different photoperiods. I found that incubating, inspecting, patrolling and perching had a treatment effect, with higher frequencies of these behaviors in colonies under a simulated seasonal photoperiod that resembles a temperate day length schedule than in colonies exposed to constant photoperiods. There was also a treatment X age effect interaction in the frequencies of five behaviors related to non-social and guarding tasks. I found no effect of photoperiod on the mean body size across treatments; however, large, older colonies tend to have lower mean worker body sizes than smaller, younger colonies. In conclusion, colonies tend to maintain a social homeostasis in the frequency of functional tasks under different photoperiod treatments. This social homeostasis is attained by fine-tuning the frequencies of individual behaviors that correspond to behaviors related to the colony developmental phase. My results provide insights on the behavioral mechanisms of social regulation in relation to environmental information in *B. impatiens*.

Key words: Bombus impatiens, photoperiod, social behavior, body size distributions.

INTRODUCTION

Photoperiod, the cyclical changes in day length due to earth's rotation, is a key environmental cue that organisms use directly or indirectly to regulate many aspects of their biology (Saunders 2002; Tauber et al. 2004). These aspects include daily and seasonal physiological changes (e.g. hormone levels), regulation of timing of key events of the individual or group life cycle (diapause, eclosion, and reproduction), and changes in social dynamics such as competition for reproduction, migration, foraging, and other behavioral responses (Pittendrigh 1960; Danilevsky et al. 1970; Gwinner 1986; Saunders 2002). The effect of photoperiod on behavior is well documented in many organisms (Nelson et al. 1990; Saunders 1997). These effects are associated with physiological and genetic responses that can be measured at the individual and the population level. For example, the flesh fly *Sarcophaga argyrostoma* is sensitive to day length thresholds below which there is maternal induction of a pupal diapause pathway (Denlinger 1971; Saunders 1982; Kenny et al. 1992). At a social level, there is a reproductive response to photoperiod in the pea aphid *Acyrtosiphon pisum* where short days activate the production of sexual forms (oviparae and males) (Johnson 1966; Lees 1989; Erlykova 2003).

Social insects also use photoperiodic cues to regulate many aspects of individual and colonial behavior (Moore and Rankin 1983; Bloch 2009). Honey bee foragers use time memory to adjust their daily and seasonal foraging schedules (Beling 1929; von Frisch 1967), and foragers also use a time compensated sun compass to detect and communicate the exact location of a resource to their nest mates (von Frisch 1967). Seasonal effects on colony reproduction are also known for many social insects that can

be directly or indirectly linked to the annual changes in day length and its environmental correlates (temperature, humidity, food availability). In the context of photoperiodic cues, individuals have evolved an endogenous circadian clock that allows them to adjust and regulate internal metabolic and behavioral processes. The molecular basis of this endogenous clock has been studied in the honey bee, *Apis mellifera*, the bumble bee *Bombus terrestris*, and the harvester ant *Pogonomyrmex occidentalis* (Bloch et al. 2003; Shemesh et al. 2007; Ingram et al. 2009).

A particularly interesting group of social insects in which to study the effects of photoperiod at both the individual and colony levels are the primitively eusocial Hymenoptera that include bees of the genus *Bombus* and multiple genera of wasps (Wilson 1971). The colony life cycle of these primitively eusocial species is strongly associated with annual and seasonal environmental fluctuations. In bumble bees for example, most of the approximately 250 species of the genus occur in the temperate regions (Williams 1998; Cameron et al 2007; Hines 2008) where there are seasonal fluctuations. Primitively social bees in these temperate latitudes are characterized by annual colonies with seasonal changes in their life cycle. In the initial solitary phase the new queen emerges from diapause and independently begins a nest, during which the queen performs all activities (foraging and reproduction). This is followed by an ergonomic phase that is characterized by constant production of workers who perform all tasks with the exception of reproduction, although workers can produce males. At the peak of reproduction the colony switches from worker production to new queen (gyne) production. In many species this reproductive phase is characterized by an increase in aggression among and within castes. Finally, at the end of the season, the old queen and

workers die and the gynes and males leave the colony in search of mates (Sladen 1921; Alford 1975; Michener 1974).

In previous experiments using the temperate bumble bee *B. impatiens*, I have shown that changes in photoperiod significantly affect colony development, specifically in aspects such as growth, oviposition rates and brood survival. By experimentally exposing captive colonies to different photoperiods throughout the social phase of the colony life cycle, I found that colonies exposed to simulated seasonal photoperiods produce, on average, larger colonies than colonies exposed to constant photoperiods (e.g. 12:12 (LD); 24(D); 24(L); and short and long days). Similar colonies appear to use photoperiodic cues to regulate the timing of the production of reproductives. Colonies exposed to simulated seasonal photoperiods began production of gynes and males with the decreasing day length phase of the cycle whereas colonies under constant photoperiods produced reproductives at any point during the social phase of the colony life cycle. These responses likely affect the reproductive success of colonies within a population by synchronizing the emergence of males and gynes (new queens) at a local or regional scale. Similarly, I found that photoperiod appears to entrain the circadian rhythms of *B. impatiens* at the colony level. Colonies under constant darkness free run with a period close to 24 hours. Colonies also modified their daily foraging rhythms following changes in day length. These results suggest that colonies, or members of the colony, could have evolved mechanisms to perceive such environmental changes. Such abilities would be expressed in workers that are exposed to daily changes of external environmental variables such as photoperiod, temperature, humidity, and resource availability. Yerushalmi et al. (2006) found an intra-colonial distinction in the ontogeny

and expression of circadian rhythms which in bumble bees is size-related. They showed that small workers have a weak expression of circadian rhythms and normally work around the clock. On the other hand, large workers, which tend to become foragers, have a stronger expression of circadian activity cycles being more active during the day and less active at night. These two patterns of circadian activity are also detectable at the molecular level (Yerushalmi et al 2006; Bloch 2009; Weiss et al. 2009).

Do foragers transmit important environmental information to all other members of the colony? To answer this question it is important to investigate behavioral changes within the colony as a result of changes in environmental information such as photoperiod. It is known that social insects are very efficient at responding to changes in their environment. For example, in bumble bees, changes in the worker composition (forager removal) can rapidly trigger task shifts in the colony to supply the work force that the colony requires (O'Donnell 2000). Other experiments induce a colony response by manipulation of temperature (Couvillon et al. 2010), resource availability and colony nutritional status (Sutcliffe and Plowright 1990; Molet et al. 2008; Couvillon and Dornhaus 2010). Studies that have also followed the distribution of tasks among workers throughout the colony life cycle found changes in the patterns of division of labor across colony developmental stages (Honk and Hogeweg 1981; Hogeweg and Hesper 1983; Cameron 1989; O'Donnell et al. 2000; Couvillon et al. 2010). Division of labor in temperate bumble bees (*Bombus*) is based largely on morphological differences among workers with weak or nonexistent temporal polyethism (Alford 1975; Cameron 1989; O'Donnell 2000, Jandt et al. 2009; Jandt and Dornhaus 2009). Generally, there is a bimodal distribution of body size with the majority of individuals being of small body

size, and a minority being large individuals. Small bees are more likely to be in-nest workers performing mostly nursing tasks, whereas large bees tend to perform foraging and guarding tasks but can also perform in-nest tasks (brood care, cleaning and incubating) (Goulson et al. 2002; Yerushalmi et al. 2006) . However, Jandt et al. (2009) found that in *B. impatiens*, worker task specialization is relatively weak. Workers, independent of age and to some degree size, perform multiple tasks during their lifetime. However, workers show some temporal constancy in their behavioral repertoire. Similar results have been found in other bumble bee species (Free 1955; Van Doorn 1987; Cameron 1989; Cameron and Robinson 1990; Cartar 1992; O'Donnell et al. 2000).

The goals of this study are to ask what are the effects of different photoperiod regimes on behavior within the colony and the distribution of body size during the social phase of the colony life cycle. Furthermore, this study attempts to explore the effects of photoperiod on *colony* patterns of division of labor. Patterns of division of labor at the individual level are described for several species of bumble bees (Yerushalmi et al. 2006; Jandt and Dornhaus 2009; O'Donnell et al. 2000). Heretofore, I have shown changes in colony development (colony size), reproduction, and colony circadian rhythms as an effect of photoperiod. I hypothesize that these changes reflect differences in behavior at the colony level. I predict that colonies exposed to different photoperiod treatments will show differences in the distribution of worker tasks by adjusting the rates of performance of specific behaviors throughout the social phase of the colony's life cycle according to the developmental stage of the colony. For example, colonies exposed to constant photoperiods can produce males and gynes at any time during the life cycle; consequently, I expect that aggression levels should increase in these colonies at earlier

time points than in colonies exposed to seasonal photoperiods where the production of reproductives is timed toward the end of the colony life cycle. Similarly, colonies exposed to a seasonal photoperiod have a longer ergonomic phase (growth) with steeper rates of worker production than colonies under constant photoperiods. In these colonies, brood care and foraging rates should be higher at earlier points than in colonies exposed to constant photoperiods. To test this hypothesis, I experimentally exposed colonies of *B. impatiens* to different artificial photoperiods and evaluated the colony responses in terms of changes in the distribution of tasks during the colony life cycle. Specifically, (1) I investigated the effect of photoperiod on frequencies of individual behaviors and on the distribution of task allocation at multiple sample points across the colonies' social phase; (2) I investigated the effect of photoperiod on circadian expression of individual behaviors; and (3) I investigated the effect of photoperiod treatments on worker size classes and body size task specialization at multiple sample points across the social phase of the colony's life cycle.

MATERIALS AND METHODS

Commercial *B. impatiens* colonies obtained from Koppert Biological Systems consisted of one queen, a few workers, and brood at various stages of development (Koppert colony research type). Each colony was transferred from its transportation box from the supplier to a wooden observation nest-box with a clear plastic top and a dark removable cover that blocked the entrance of light to the nest-box, thus simulating natural conditions (Figure 1). The nest-box was composed of a main chamber (25x25x15 cm) where the colony was placed, and a smaller chamber (8x25x15 cm) where individuals

transitioned between the nest and the foraging area which was used by the bees for defecation. The foraging area consisted of a mesh cage on a wooden frame (75x100x75 cm), and was connected to the nest box with a 25x3 cm transparent plastic tube that the bees could walk through.

All colonies were fed fresh pollen supplied from local apiaries and honey water solution provided by Koppert (methodology according to Plowright and Jay 1966; Cameron 1989; Cnaani et al. 2000). Pollen grains were ground and mixed with honey water solution to produce a homogeneous paste added directly to the colony box. Pollen was added daily with a randomized time schedule to prevent the colony synchronization to a feeding schedule. To avoid starvation or over-feeding, the amount of pollen was normalized to the progression of colony growth (Evans et al. 2007). The honey water solution was provided once a day in the foraging area using 10 ml transparent plastic vials hung from the roof of the foraging area. Each vial had two to four small holes at the base from which workers were able to extract the solution. Honey water daily volume was also adjusted to track the colony growth.

Experimental Design

To investigate changes in worker division of labor over time as an effect of photoperiod, I randomly assigned nine colonies of *B. impatiens* to one of three photoperiod treatments (three colonies per treatment) that have shown in previous studies to have the highest impact in terms of population growth, timing for the production of reproductives and individual and colony daily patterns of activity (Hernandez in prep). The first photoperiod treatment, a simulated natural photoperiod, was designed using

annual day length values similar to the St. Louis region (maximum total light is 14h 52min on the longest day of the year). Light treatment began at 12 h 45 min total light (approximating the beginning of the social phase in the colony life cycle), and day length was increased by 15 minutes every fifth day until reaching a maximum of 15 hours of light followed by a decrease of day length until the end of the experiment (Figure 2) The second and third photoperiod treatments consisted of constant 12L:12D (Light:Dark), and a constant 24 DD photoperiod. Colonies were maintained under constant temperature (28°C) and humidity (50% relative humidity) (Duchateau and Velthuis 1988) in 3 isolated rooms of the Animal Care Facility at the University of Missouri-St. Louis.

Data acquisition

I quantified behaviors and body size of individual bees at six time points during the social phase of the colony's life cycle starting at day 15 from the beginning of the experiment and again every 15 days until day 90 (Figure 2) which included the natural phases of a colony population growth curve as presented previously on this species by Hernandez (in prep) (Figure 2) and in other bumble bee species (Honk and Hogeweg 1981; Cameron 1989). Video recordings of colony activity were taken using a camcorder Sony (DCR-HC96) using the Nightshot® setting allowing recording in the dark with infrared light. Each colony was recorded for 10 minutes twice a day, 12 hours apart to include the subjective scotophase (night time) and the subjective photophase (day time). The order of video recording of the colonies was randomly selected at each video session. Colony nest boxes were kept in constant dark at all times (including the video recording sessions) thus simulating their natural nesting habits. At the beginning of the

experiment I individually marked workers on the thorax with a number tag (The Bee Works, Ontario, CA). These bees were measured for body size and used as reference for measurements of body sizes of bees that emerged after the start of the experiment. Bees that emerged after of the colony was transferred to the nest box were not marked, because marking bees during the observation periods requires a constant disturbance to the colony which could have affected normal behavior. For each video I measured individual body size by calculating the inter-tegular distance as a proxy for body size (Goulson 2002; Jandt et al. 2009).

I used scan sampling (Altmann 1974) to record behaviors performed by all bees present in the colony every 5 minutes for a total of three counts of activity per session (O'Donnell 2000). I recorded social and non-social activities using the ethograms modified from Gamboa et al. (1987), Cameron (1989), and O'Donnell et al. (2000). I included the non-social activities of self-grooming (SG), resting (RE), pulling cotton (PC), eating pollen (EP), drinking honey (DH), walking (WA), and foraging (FO). Social interactions were divided between brood care activities (feeding larvae (FL), incubation (IB), fanning (FA), scraping wax (SW), inspecting (IN) (which includes working in honey pots and quick antennal inspection to the brood)); Guarding activities (patrolling (PA), perching (PE)); and agonistic behaviors including biting, chasing, and charging all included into one aggression behavior (AG) (See Table 1 for a detailed explanation for each behavior).

Statistical analysis

All statistics were produced on JMP (2001) and in R (R Development Core Team 2004). For each colony I calculated the relative probabilities of behavior performance. I obtained the probability of a particular size class to engage in a particular task or group of tasks following the calculations described by Seeley (1982). Several studies have shown that task allocation is strongly associated with body size but not age in bumble bees (Brian 1952; Jandt and Dornhaus 2009). For this reason probabilities of behavior performance were calculated using body size classes instead of age classes. To determine body size classes I used the quartile values of the body size distributions obtained for each video session. The four body size classes obtained were used for a Classificatory Discriminant Analysis (CDA) using the forward stepwise method to identify the main tasks that best explained the variance in the canonical space of the different photoperiod treatments. The significance of the multivariate test was obtained by the Wilks' lambda.

Normality for each body size distribution was analyzed using descriptive statistics and the Shapiro-Wilk W test (Zuur et al. 2009). Spearman rank correlation was used when the assumptions of parametric tests were not met. I performed a generalized linear mixed model test that allows for repeated measurements to explore the effect of photoperiod on the body size distributions over time using the different sampling points as a random factor and the photoperiod treatment as fixed factors, and using an autocorrelative matrix of covariance that allows higher variance correlation among closer time points (Zuur 2010). Similarly, the effect of photoperiod on the type of task

performed was also tested using a General Linear Mixed Model (GLMM) using tasks as a treatment factor or as a response variable where each task was tested independently.

RESULTS

Behavior

The classificatory analysis showed a clear separation of the three photoperiod treatments based on the relative variance of the behaviors evaluated, with the first two canonical components explaining over 80% of the variation (CDA Wilk's lambda $p=0.0002$)(Figure 3). Behaviors such as patrolling and aggression were more closely associated with the simulated seasonal photoperiod treatment, whereas the 24D and 12L:12D treatments were more closely associated with brood care and most non-social activities. The proportion of functional tasks (brood care, non-social and guarding tasks) remained relatively similar in all three treatments throughout the colonies' life cycle (Figure 4). When behaviors are examined individually, however, there were significant treatment differences in the relative proportion of four individual behaviors across the colonies' life spans (Figure 5; Table 2).

There were significant differences in the frequency of incubating (IB) across treatments ($F=10.9292$, $p<0.0001$). The *post hoc* analysis revealed IB in the 24D photoperiod treatment was significantly more frequent than in the 12L:12D and the simulated seasonal photoperiod treatments (Tukey's multiple comparison test $p<0.05$). The highest frequency of incubation in the 24D photoperiod treatment occurred between day 45 and 60 after the beginning of the experiment similar to the 12L:12D treatment.

The simulated seasonal photoperiod, on the other hand, did not show differences over time in the frequencies for this behavior (Figure 5 IB).

The simulated seasonal photoperiod treatment resulted in significantly more frequent inspecting (IN) behavior compared to the constant photoperiod treatments ($F=8.3869$, $p=0.0003$). There were no differences in the frequency of inspections over time for the two treatments with a photophase (Figure 5 IN) (Tukey's multiple comparison test $p<0.05$).

There were significant differences in the overall frequencies for the two guarding behaviors analyzed (patrolling (PA) and perching (PE)) across treatments and over the social phase of the colony life cycle (Figure 5 PA; PE) (PA $F=9.1556$, $p=0.0001$; PE $F=9.1556$, $p=0.0001$). Colonies in the simulated photoperiod treatment showed higher frequencies of patrolling than the two constant photoperiod treatments according to the *post hoc* test. There was an increase in the frequency of the patrolling behavior at day 60 for the seasonal photoperiod treatment that was not present in for the two photoperiod treatments. On the contrary, *post hoc* analysis revealed that perching was significantly more frequent for the 24D treatment (Tukey's multiple comparison test $p<0.05$), especially at the two tail ends of the sampled periods. The simulated seasonal photoperiod showed a constant proportion of perching across time.

Rates of self grooming (SG) and fanning (FA) were not different between photoperiod treatments, but these behaviors showed significant differences as a function of colony age (SG $F=5.6605$, $p<0.0001$, FA $F=8.1937$, $p<0.0001$).

There was an effect of the interaction of photoperiod treatment and colony age for the frequencies of resting (RE) ($F=2.7931$, $p=0.0024$), pulling cotton (PC) ($F=2.9172$,

$p=0.0015$), scraping wax (SW) ($F=3.0746$, $p=0.0009$), patrolling (PA) ($F=6.7211$, $p<0.0001$), and perching (PE) ($F=3.0528$, $p=0.0010$). The simulated seasonal and the 12L:12D photoperiod showed high frequencies of resting (RE) at the beginning of the experiment with a subsequent decrease as the colony aged. On the other hand, the 24D photoperiod treatment showed a significant increase in the frequency for this behavior at day 45 that was maintained at high levels thereafter. Pulling cotton (PC) showed relatively similar frequencies for all of the photoperiod treatments over time, except for the last time point (day 90) where there was a significant increase in the frequency of this behavior for the 12L:12D photoperiod treatment. Scraping wax (SW) was a behavior that showed a high frequency in all three photoperiod treatments (10% 24D; 12% 12L:12D; and 15 % seasonal photoperiod). The frequency of this behavior increased over time for the constant photoperiod treatments (24D and 12L:12D), whereas for the seasonal photoperiod this behavior was highest at the beginning of the experiment (18% at day 15) and by the end of the colony cycle it dropped to less than 8% ($X^2=16.85$; $p=0.002$ (day 15); $X^2=8.054$; $p=0.02$ (day 90); Figure 5 SW).

Aggressive behaviors (AG) had different frequency patterns across treatments. The absence of significant differences among photoperiod treatments could be due to small sample size for this behavior (less than 1% of observed behaviors in all treatments; Figure 5 AG).

Body size

Classificatory discriminant analysis revealed a worker size-class separation among the different photoperiod treatments (CDA Wilk's lambda $p<0.0001$). The two

constant photoperiod treatments did not show significant distances from one another within the canonical space, but the simulated seasonal photoperiod differed from them, primarily along the first canonical axis (Figure 6). In the CDA, the simulated seasonal treatment was more closely grouped with the largest body size classes (classes three and four) and consequently with the tasks more closely correlated with large body sizes, which are foraging, guarding and non-social tasks (Figure 6). The two smaller size classes were more closely associated with the constant photoperiods and with brood care tasks, especially larval feeding and incubation as well as perching (Figure 6).

The GLMM analysis revealed that only drinking honey (DH) ($F=5.5229$, $p=0.0010$) and foraging (FO) ($F=3.1612$, $p=0.0002$) were significantly different as an effect of body size. *Post hoc* analysis showed that the largest body sizes showed higher frequencies of performing such behaviors compared to the smaller size classes (Tukey's multiple comparison test $p<0.05$) (Table 2).

There was no effect of photoperiod on the overall mean worker size distribution (ANOVA $F=1.1681$; $p=0.1215$), however there is a significant effect of photoperiod on the body size distributions when the data are analyzed temporally (GLMM $F=6.6998$; $p<0.0001$). Overall, there is a general tendency toward a reduction in the mean body size over time with a final increase at the last time point (Figure 7). In the simulated photoperiod treatment there was a significant reduction of the mean body size as the colony ages ($r^2 = 0.2$, $p<0.0001$). This was not the case for the two constant photoperiod treatments where there is no relationship between average body size change and colony

age (24D $r^2 = 0.002$, $p=0.1403$; 12L:12D $r^2 = 0.0041$, $p=0.8940$). Unlike the seasonal photoperiod treatment, colonies in the constant photoperiod treatments did not increase in size as much during the colony's life cycle thus I did not find a significant relationship between colony size and the change in body size through time (24D $r^2=0.0035$, $p=0.823$; 12L:12D $r^2=0.0051$, $p=0.921$)

The shape of the body size distribution for the three photoperiod treatments was positively skewed (Figure 8). Normality tests show that colonies shift from a relatively normal distribution earlier in the colony development to a more complex higher positively skewed non-normal distribution toward the end of the colony life cycle (Table 3). Skewness is positively associated with colony size but not age (Colony size $r^2=0.08$; $F=4.56$; $p=0.037$; Colony age $r^2=0.005$; $F=0.26$; $p=0.60$), whereas kurtosis is positively associated to both (Colony size $r^2=0.18$; $F=11.289$; $p=0.0015$; Colony age $r^2=0.12$; $F=7.17$; $p=0.010$). Standard deviation of body size, on the contrary, is negatively associated with size but not age (Colony size $r^2=0.10$; $F=5.84$; $p=0.019$; Colony age $r^2=0.028$; $F=1.41$; $p=0.240$).

Circadian patterns of behavior expression

There was no time of day effect on the frequencies of the pooled non-social, brood care and guarding behaviors over the social phase of the colony life cycle in any of the photoperiod treatments ($F=1.211$ $p=0.845$). Among the individual behaviors, only eating pollen ($F=4.1555$ $p=0.0421$) and foraging ($F=6.6045$ $p=0.0015$) showed a significantly higher probability of being performed during the photophase period of the

day for both of the treatments with a photophase. A similar pattern was observed for these two tasks in the 24D photoperiod although there is no photophase for this treatment.

Circadian patterns of size distribution

There was a significant difference in the average body size within the colony between day and night distributions for the simulated natural photoperiod and the 24D treatments, but not in the 12L:12D photoperiod treatment (Figure 9; ANOVA $F=12.22$; $p=0.0001$, *post hoc* Tukey's multiple comparison test). Colonies exposed to a seasonal photoperiod had a higher mean body size worker population inside the nest during the night than during the day. Colonies exposed to 24D also showed mean body size differences at a 12 hour interval, but because these colonies were exposed to constant dark there was no distinction between day and night. Colonies exposed to a constant 12L:12D showed no difference in the mean body size between the subjective day and night (Table 4).

DISCUSSION

Individual behaviors and colony-level task allocation

Social insect colonies show behavioral flexibility in task allocation that permit a response to environmental fluctuations. For example, Pendrel and Plowright (1981) showed in *B. terrestris* that experimental manipulation of the worker composition from a particular functional class (e.g. foragers) activates an immediate change in the allocation of tasks by recruiting workers that were performing other tasks and at the same time

increasing the rates at which the task (foraging) was performed. The results of my experiment showed the ability of a colony to maintain a constant ratio of worker task allocation over the social phase despite major differences in an environmental cue, differences in day length. Photoperiodic cues were sufficient to significantly modify the performance frequency of four individual behaviors and the interaction between photoperiod and colony age for five behaviors. Despite these significant effects attributable to photoperiod treatment differences, colonies maintained a relatively constant proportion of functional tasks (brood care, nest maintenance, guarding) across the full social phase of the bees' life cycle.

Homeostasis in social insects has been extensively studied. For example, there is ample evidence that colonies self-regulate their internal nest environmental conditions such as temperature, humidity, and circulating gas levels (Simpson 1961; Stabenthienner et al. 2010). Results reported here reveal social homeostasis in the colony by maintaining a relatively stable net ratio of functional tasks under three photoperiod regimes across the social phase of the colony life cycle. The net ratio of functional tasks was maintained despite significantly different frequencies of individual behaviors and significant interactions between colony age and photoperiod treatments. In previous experiments, Hernandez (in prep) reported significant increase in colony size in colonies exposed to a seasonal photoperiod beginning approximately between days 30-40 from the beginning of the experiment (Figure 10). Because all colonies from different photoperiod treatments were similar in size at the start of the experiment, I did not expect to encounter different proportions of functional tasks in different photoperiod treatments before day 30-40.

However, it was surprising to find that the distribution of functional tasks (brood care, non-social, and guarding tasks) through time was consistent across treatments and sampled points. This result suggests that colony size has no effect on the colony-level distribution of worker tasks.

I did not follow individual bees during the experiment. Such analyses have been done for *B. impatiens* (Jand and Dornhaus 2009), *B. agrorum* (Brian 1952), *B. terrestris* (Pendrel and Plowright 1981; Honk and Hogeweg (1981), *B. griseocollis* (Cameron 1989), and *B. nefarious* (O'Donnell 2000). Such studies have demonstrated the absence of complete specialization of tasks for workers, with workers of all ages capable of performing all tasks within the colony.

Body size effects

The worker caste in bumble bees exhibits size polymorphism with variations in total mass that in some species can span a 10- fold difference (Goulson 2002; Couvillon et al. 2010). It has been determined that there is a tendency of workers of certain class sizes to show a degree of specialization in particular tasks.

The present study suggests that, across the social phase, colonies tend to maintain a constant ratio of body size groups that would be responsible for functional tasks. This worker body size ratio is maintained throughout the colony life cycle as the colony increases in number. The change in number of individuals as the colony gets older does not affect the proportion of tasks performed within the colony, but only increases the number of individuals that will perform each group of tasks. The fact that photoperiod

had no effect on the mean worker size distributions is consistent with the maintenance of a constant functional task ratio over time across photoperiods. There was, however, a negative relationship between mean body size ratio and colony size, particularly in colonies exposed to the seasonal photoperiod treatment. Furthermore, older larger colonies show a slightly broader range of body sizes than colonies at young ages. A possible explanation is that as colonies become larger, feeding dynamics change over time such that feeding of brood becomes less equitable and more biased toward certain larvae. It has been shown that bees at the periphery of the nest are smaller because they receive less attention from adults (Couvillon and Dornhaus 2009). The fact that the colony becomes much larger and there are more bees to feed could reduce the amount of food per capita or perhaps the intensity of feeding from individual workers (gyne sizes were not included). As expected, there was no change in the mean size distribution of colonies under constant photoperiods, nor was there a significant change in the growth pattern. Colonies never attained sizes comparable to those from colonies under seasonal photoperiod, even though their overall patterns of distribution were the same.

Jand and Dornhaus (2009) described division of labor in *B. impatiens* colonies kept in captivity. Their colonies were kept under 10:14 Light:Dark cycles and the nest was exposed to light. They found an overall body size distribution of tasks similar to those found in this experiment. Small to intermediate size bees were more likely to feed larvae and to incubate, whereas large bees were more often foragers and fanners. However, the results from their observations resemble more the task allocation obtained in my study from colonies exposed to constant photoperiods. In contrast, colonies

exposed to the seasonal photoperiod in this experiment show that large bees are more likely to feed larvae particularly early in the colony development. At older ages there is a higher likelihood of intermediate size workers feeding larvae, suggesting that late in the colony cycle the colony size contained gynes. Patterns of division of labor observed in this study matched the expected trends of colonies in the reproductive phase of their social cycle, e.g. elevated rates of aggression, brood care and patrolling tasks and lower level of other non-social cues. These patterns are similar to those found in natural colonies under a seasonal photoperiod, the only difference being the timing at which the frequency of these tasks is performed, which in these colonies occurs late in the life cycle.

Circadian patterns

The internal environment within a *B. impatiens* nest is of constant climate and dark conditions, which could facilitate the around the clock patterns of task distribution documented in this experiment. Only foraging-related behaviors showed circadian differences, with lower levels of expression during the subjective night. Interestingly, rhythmic foraging related tasks were maintained in colonies exposed to constant darkness. In previous experiments I found that even in the absence of photoperiod information, colonies maintain an endogenous colony rhythm of activities that free runs with a period close to 24h. These results suggest that the colony has an endogenous circadian clock which synchronizes (or entrains) foragers to maintain a circadian expression of their task. Further analysis should explore clock gene expression profiles of foragers under constant dark conditions to determine whether such individuals also

maintain an endogenous circadian molecular clock as observed in individuals under normal circadian photoperiods (Yerushalmi et al. 2006).

CONCLUSIONS

Research presented here provides data on frequencies of individual behaviors, task allocation, and body size distributions at different points across the social phase of colonies of the bumble bee *Bombus impatiens* reared in captivity under three photoperiod regimes. I document changes in the frequency of individual behaviors, task allocation, and the individuals, grouped by body size classes that are more likely to perform the behaviors and tasks.

The significance of this study in the larger picture of the regulation of social insect colonies has been to show how behavioral flexibility and the coordination of task allocation can contribute to social homeostasis. Homeostasis is an emergent property of collective behavior of individuals in a colony. Results presented here demonstrate task homeostasis under three experimental photoperiod treatments across the full span of the social phase of the life cycle despite significant differences in the expression of individual behaviors among treatments. Body size analysis revealed that under different photoperiod conditions colonies maintain stable worker body size ratios that are associated with task allocation over the social phase of the colony's life cycle. This research therefore provides insights on the behavioral mechanisms of social regulation in relation to environmental information in *B. impatiens*.

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Table 1. list of behaviors recorded during the observations. The behaviors are divided among four functional classes.

Non-Social	Behavior
RE (Resting)	A bee is immobile for more than 30 seconds with the antenna or the abdomen resting on the substrate.
EP (Eating pollen)	A bee is consuming pollen from pollen cells or pollen that has been directly provided by the researcher.
DH (Drinking honey)	A bee (that has not returned from the foraging area) is observed in the honey pots for several seconds; these bees contract the abdomen characteristic of honey intake.
WA (Walking)	A bee is observed rapidly moving on the brood area. The antennae are not touching the brood or provision cells. This behavior could be a transition from one behavior to the next.
PC (Pulling cotton)	Workers construct an insulation roof by tearing cotton using their mandibles and accommodating it using their legs.
SG (Self grooming)	Individuals use their legs to groom some part of the body.
Brood care	
IB (Incubating)	A bee wraps its body around a cocoon extending and flattening its abdomen to maximize the contact area. In some cases abdominal pumping is observed during the incubation period.
FA (Fanning)	Rapid movement of the wings while in a stationary position.
FL (Feeding larva)	Bee regurgitating food into a larval cell. A bee opens a hole at the center of the cell and proceeds to feed the larvae followed by closing the orifice.
IN (Inspecting)	A bee moves through the brood area touching cells with its antennae.
SW (Scraping wax)	A bee removes wax from cocoons and other cells. This behavior is characterized by quick up and down movements of the head while using its mandibles to remove the wax.
Guarding	
PA (Patrolling)	A bee walks on the periphery of the brood with their antennae raised sometimes leaving the brood area but returning rapidly.
PE (Perching)	A bee stands with its antennae raised and the legs in an extended position for several seconds.
FO (Foraging)	A bee is observed returning from the foraging area and depositing honey in a honey cell.
AG (Agonistic)	A bee is observed biting, lunging other bee normally displaying an aggressive posture.

Table 2. *p* value results from the GLMM where each behavior is evaluated for treatment, colony age classes, worker body size, treatment X colony age interaction and treatment X worker size interaction. Bonferroni's corrected alpha of 0.0055 was used as the significance criterion. Significant values are represented in bold.

	Photoperiod	Colony Age	Worker Size	Photoperiod X colony age	Photoperiod X Worker size
Non-Social					
RE (Resting)	0.0135	0.0238	0.3872	0.0024	0.4146
EP (Eating pollen)	0.2847	0.0412	0.7304	0.2730	0.0879
DH (Drinking honey)	0.0944	0.0076	0.0010	0.1581	0.8974
WA (Walking)	0.3975	0.0325	0.8192	0.5419	0.6613
PC (Pulling cotton)	0.1099	0.0803	0.5843	0.0015	0.0737
SG (Self grooming)	0.2356	<0.0001	0.7508	0.0380	0.5493
Brood care					
IB (Incubating)	<0.0001	0.0011	0.3130	0.1335	0.9395
FA (Fanning)	0.0158	<0.0001	0.1557	0.8513	0.2170
FL (Feeding larva)	0.0993	0.0193	0.5366	0.7997	0.2197
IN (Inspecting)	0.0003	0.0047	0.5361	0.5166	0.7277
SW (Scraping wax)	0.0462	0.0137	0.1159	0.0009	0.0254
Guarding					
PA (Patrolling)	0.0007	<0.0001	0.0246	<0.0001	0.7187
PE (Perching)	0.0001	<0.0001	0.9403	0.0010	0.8949
FO (Foraging)	0.1164	0.2204	0.0002	0.0618	0.7194
AG (Agonistic)	0.1420	0.3372	0.9897	0.4405	0.3499

Table 3. p-values from Shapiro-Wilk's normality tests. *H₀* is that mean colony body size distributions fits a normal distribution. Data are displayed at the overall colony worker body size distribution and the colony body size distribution at each of the six sample points measured. All 9 colonies were analyzed separately; therefore, Bonferroni corrected alpha of 0.0055 was used as the significance criterion. Significant values are represented in bold.

	Overall	15	30	45	65	75	90
1	<0.0001	0.0104	0.4011	0.0595	0.0002	0.4982	N/A
2	<0.0001	0.0985	0.0104	0.0001	0.0387	0.0002	<0.0001
3	<0.0001	0.0029	0.8473	0.6766	0.6151	0.3934	0.0002
4	<0.0001	0.0051	0.0003	0.0085	0.6088	0.0064	0.0025
5	<0.0001	0.0085	0.1802	0.064	<0.0001	0.0017	0.0003
6	0.0008	0.0114	0.6069	0.0101	0.6231	0.0126	N/A
7	<0.0001	0.0037	0.4515	0.002	0.0003	<0.0001	<0.0001
8	<0.0001	0.1849	0.1717	0.0579	<0.0001	0.0152	<0.0001
9	<0.0001	0.00001	<0.0001	0.0013	0.0002	<0.0001	0.0002

Table 4. Photophase-scotophase differences in the colony mean body size over all sample periods for each of the nine study colonies. Values are mean \pm SD. *p* value results from the ANOVA test. All 9 colonies were analyzed separately, therefore, Bonferroni corrected alpha of 0.0055 was used as the significance criterion. Significant values are represented in bold.

Treatment	Colony	Photophase (Subjective day)	Scotophase (Subjective night)	<i>p</i> value
24 DD	1	4.482 \pm 0.65	4.268 \pm 0.51	0.0031
	2	4.718 \pm 0.52	4.387 \pm 0.44	0.0001
	3	4.473 \pm 0.46	4.283 \pm 0.47	0.0008
12L:12D	4	4.361 \pm 0.49	4.596 \pm 0.56	<0.0001
	5	4.579 \pm 0.48	4.473 \pm 0.55	0.05
	6	4.493 \pm 0.54	4.530 \pm 0.52	0.6223
Seasonal Photoperiod	7	4.683 \pm 0.53	4.526 \pm 0.53	0.0006
	8	4.518 \pm 0.51	4.620 \pm 0.51	0.0242
	9	4.480 \pm 0.50	4.296 \pm 0.50	<0.0001

FIGURE LEGENDS

Figure 1. Still image captured from one of the videos obtained using the Nightshot® setting to avoid exposure of light to the colony. Note the marked queen located on the upper right corner of the image.

Figure 2. Population growth curve for an idealized temperate bumble bee colony. The numbers below the curve show the 6 time points used for collecting data. The top axis presents the day length of light hours (photophase) that was applied to the seasonal photoperiod treatment.

Figure 3. Classificatory Discriminant analysis (CDA) comparing the influence on 15 behaviors of three photoperiod regimes (simulated seasonal, 12L:12D, and 24D). Green lines show the correlation for each behavior in the canonical space. The behaviors are: self-grooming (SG), resting (RE), pulling cotton (PC), eating pollen (EP), drinking honey (DH), walking (WA), foraging (FO), feeding larvae (FL), incubation (IB), fanning (FA), scraping wax (SW), inspecting (IN), patrolling (PA), perching (PE), and agonistic behaviors (AG). Red circles represent the ordination of the three photoperiod treatments in the canonical space.

Figure 4. Percentages of functional task categories for each of the three photoperiod regimes obtained at each of the six sample points throughout the social phase of

the colonies' life cycles. Task categories from top to bottom of each histogram match the top to bottom placement of the codes at the right of the figure.

Figure 5. Relative frequency of performance in three photoperiod regimes for each of the individual behaviors measured in the analysis. The behaviors are: self-grooming (SG), resting (RE), pulling cotton (PC), eating pollen (EP), drinking honey (DH), walking (WA), foraging (FO), feeding larvae (FL), incubation (IB), fanning (FA), scraping wax (SW), inspecting (IN), patrolling (PA), perching (PE), and agonistic behaviors (AG). Black boxes group behaviors into functional classes. Asterisks represent behaviors that show significant differences among photoperiod treatments. For the photoperiod treatments: triangles = simulated seasonal photoperiod; squares = 12L:12D; and diamonds = 24D.

Figure 6. Classificatory Discriminant analysis (CDA) comparing the influence of each task measured on the interaction between the three photoperiod treatments (blue circles) and the four body size classes (red numbers 1-4) defined using quartiles obtained from the body size distributions for each photoperiod treatment (Red circles). Green lines show the correlation for each one of the tasks variables used for the analysis in the canonical space. Behavior codes are: self-grooming (SG), resting (RE), pulling cotton (PC), eating pollen (EP), drinking honey (DH), walking (WA), foraging (FO), feeding larvae (FL), incubation (IB), fanning (FA), scraping wax (SW), inspecting (IN), patrolling (PA), perching (PE), and agonistic behaviors (AG).

Figure 7. Mean worker body size at different sample points over the course of the social phase in colonies exposed to three different photoperiod treatments. Error bars represent standard errors.

Figure 8. Body size distributions for the three photoperiod treatments (A) 24 D; (B) 12L:12D; and (C) simulated seasonal photoperiod. Note the positive skewed long tails. None of the distributions fit a normal distribution (Shapiro-Wilk's normality test $p > 0.05$)

Figure 9. Mean body size (thorax length) for the three photoperiod treatments.

Measurements were made in photophase and scotophase for simulated seasonal (Seasonal) and 12L:12D treatments and at corresponding times for the 24D treatment. Lower case letters show the significant groups obtained by the ANOVA *post-hoc* test. Categories that share the same letter show no significant differences in the colony mean body size at values of $p > 0.05$.

Figure 10. Colony population size over the social phase of the colony's life cycle for the three photoperiod treatments. For the photoperiod treatments: triangles = simulated seasonal photoperiod; squares = 12L:12D; and diamonds = 24D. Values were normalized by the initial population size.

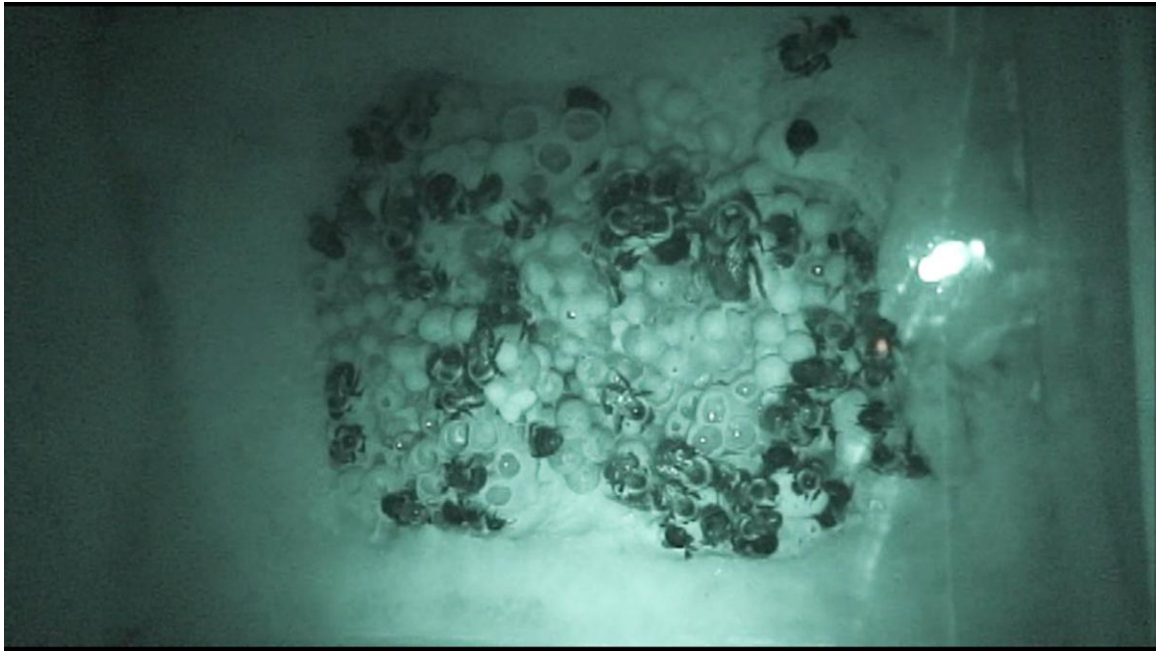


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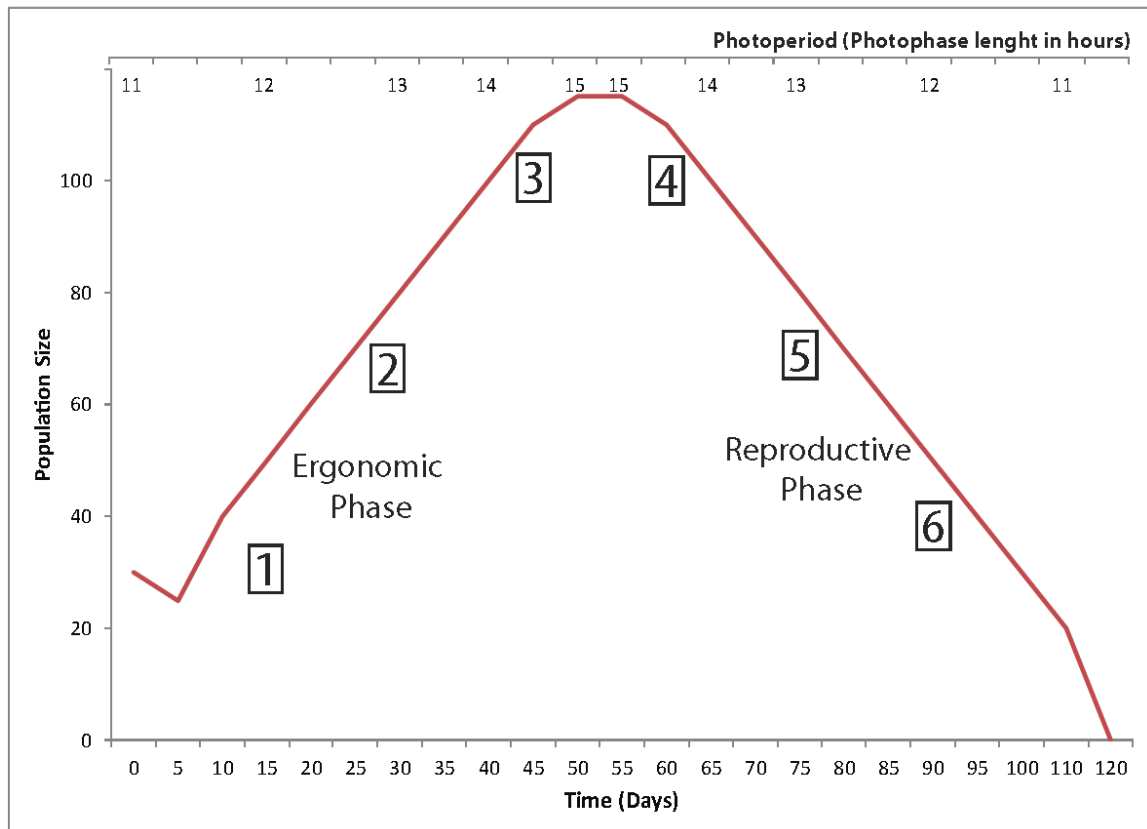


Figure 2

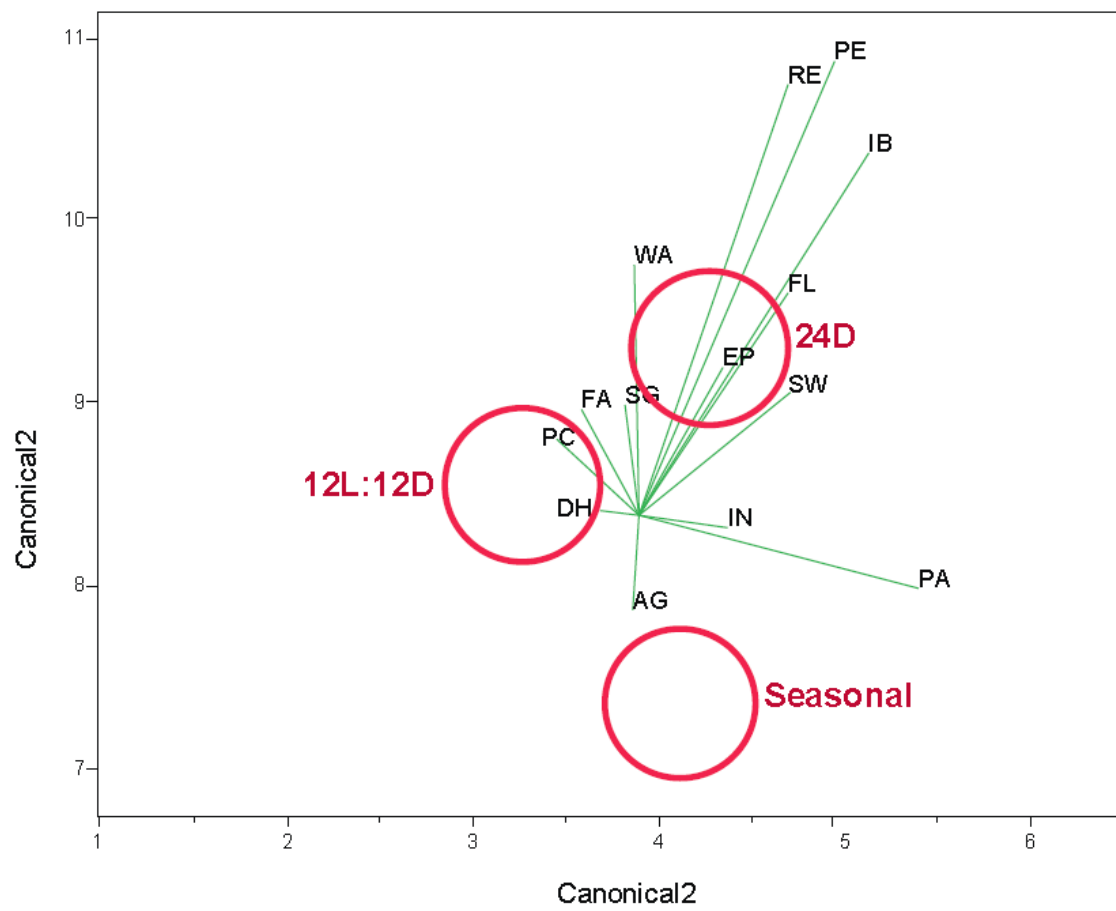


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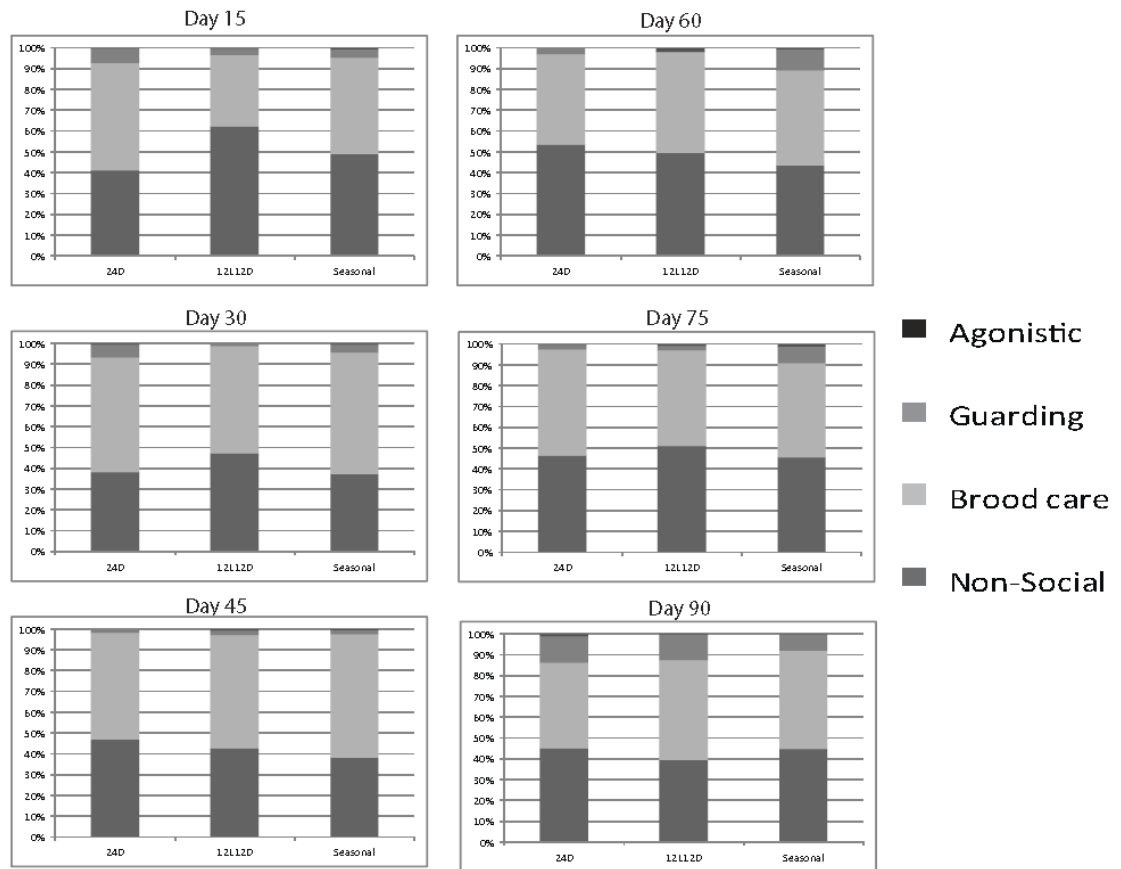


Figure 4

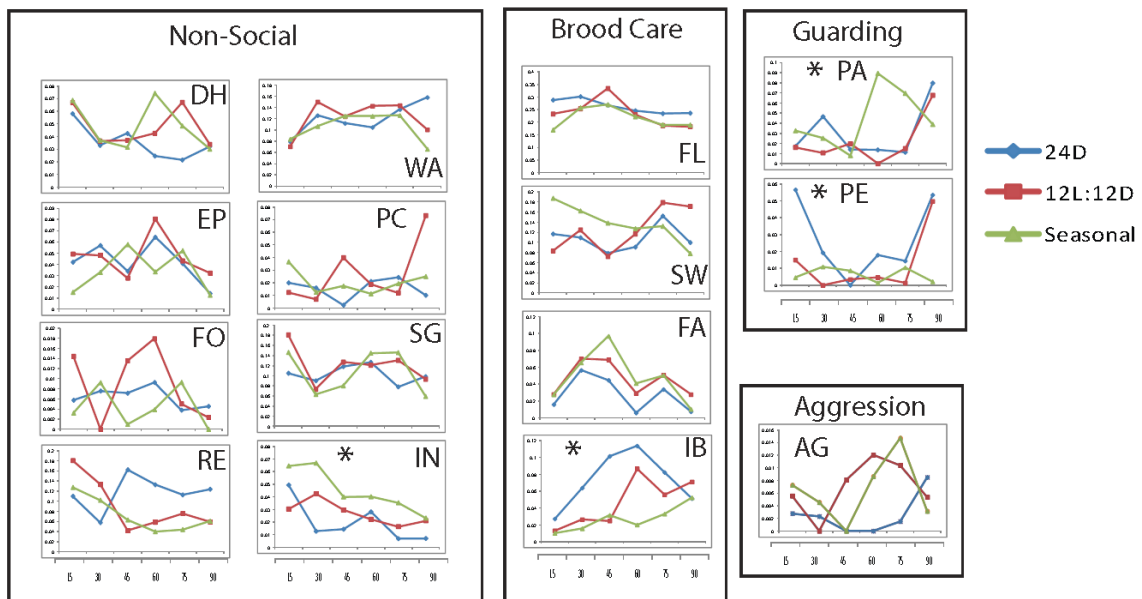


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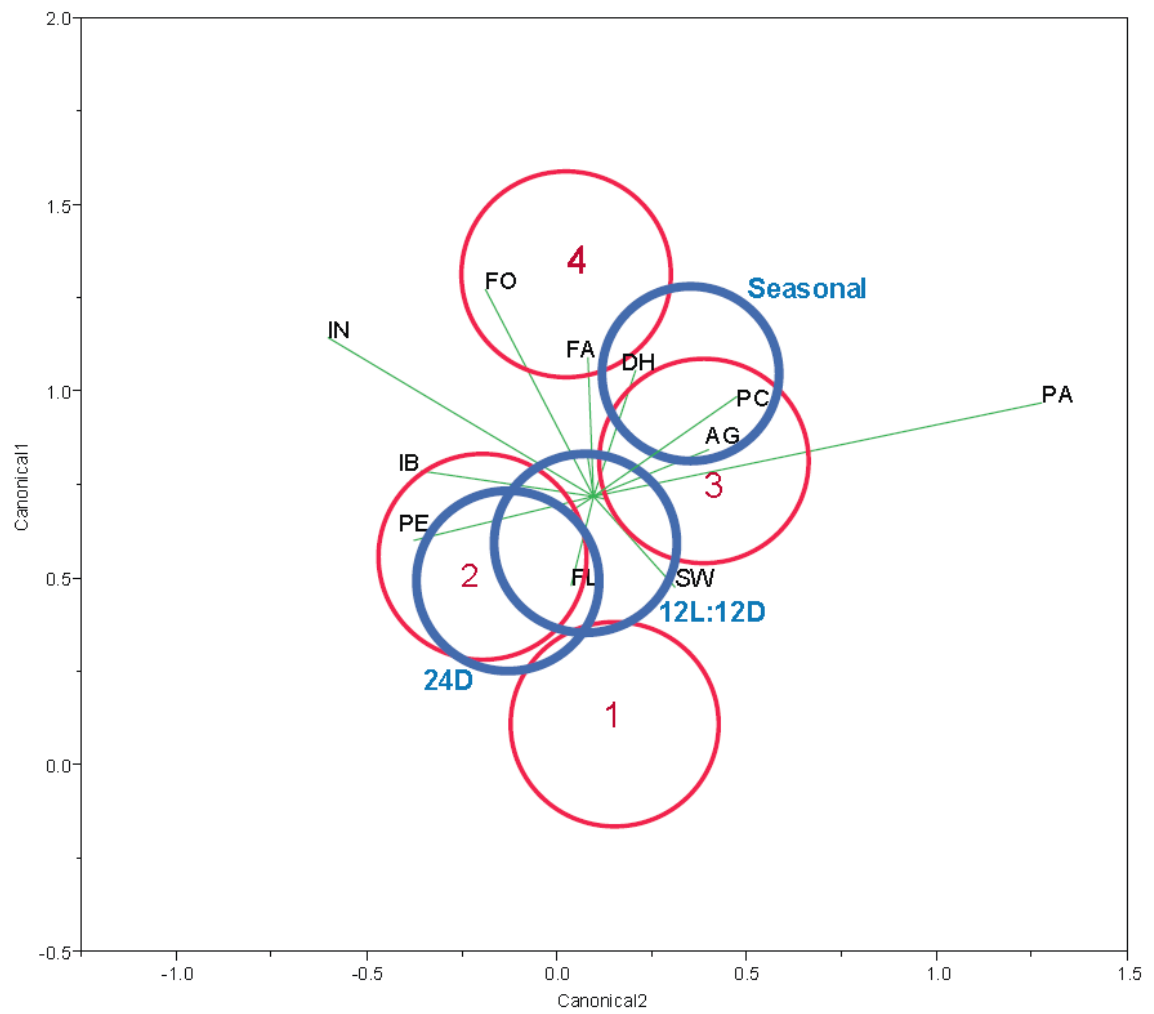


Figure 6

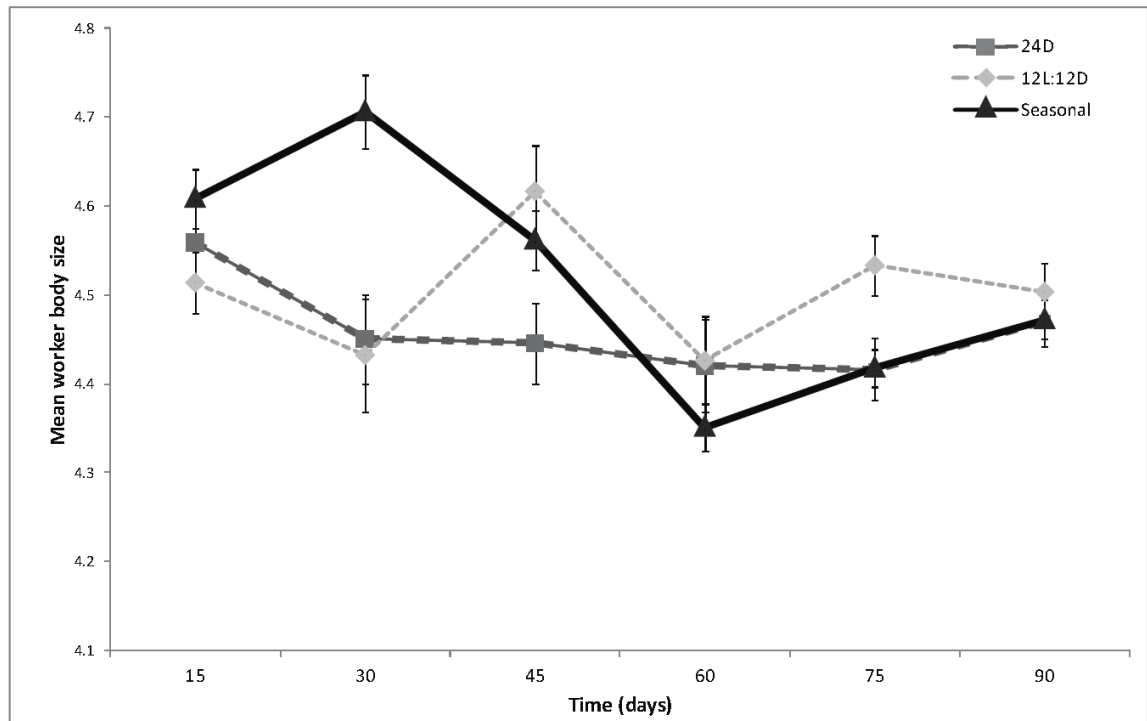
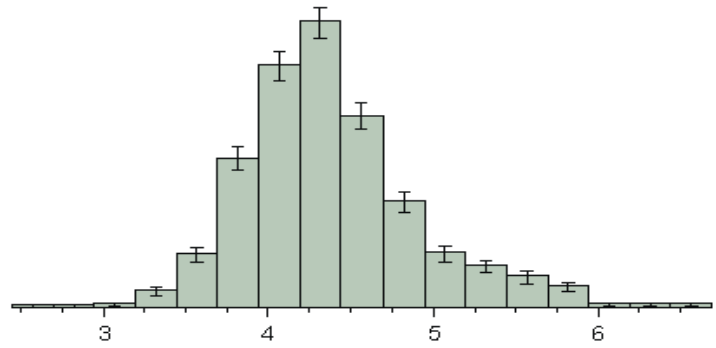
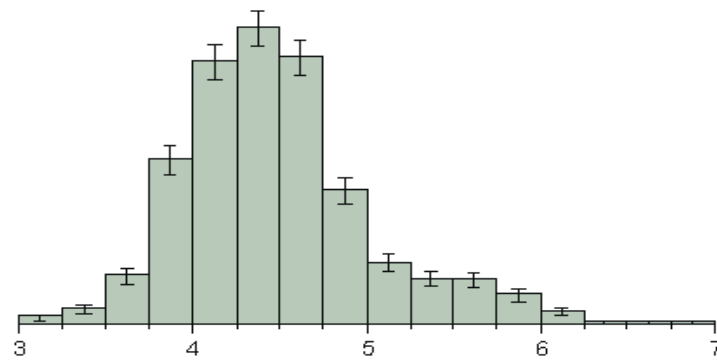


Figure 7

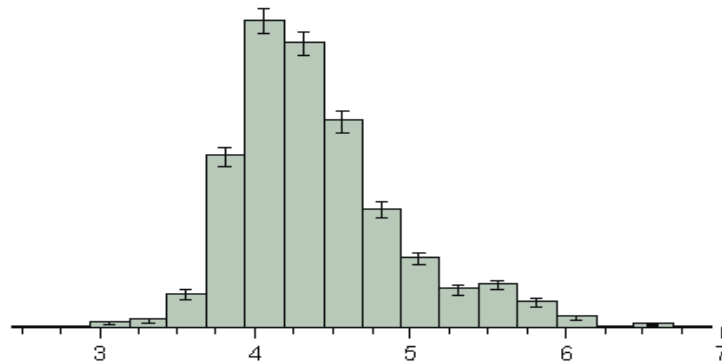
A



B



C



Thorax Width (mm)

Figure 8

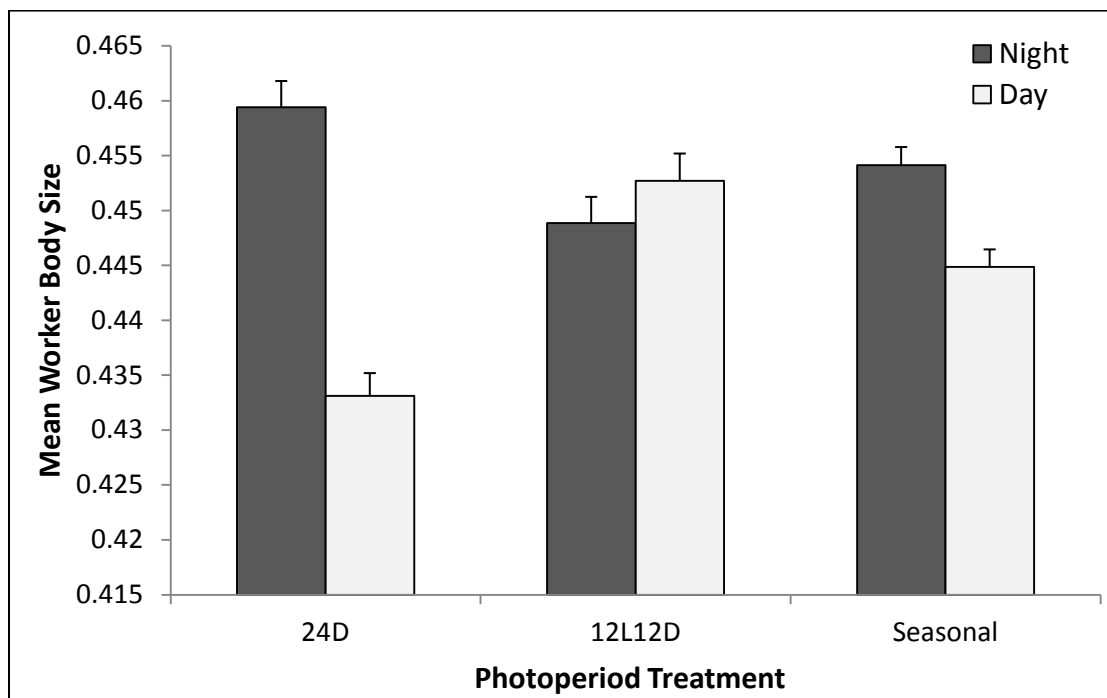


Figure 9

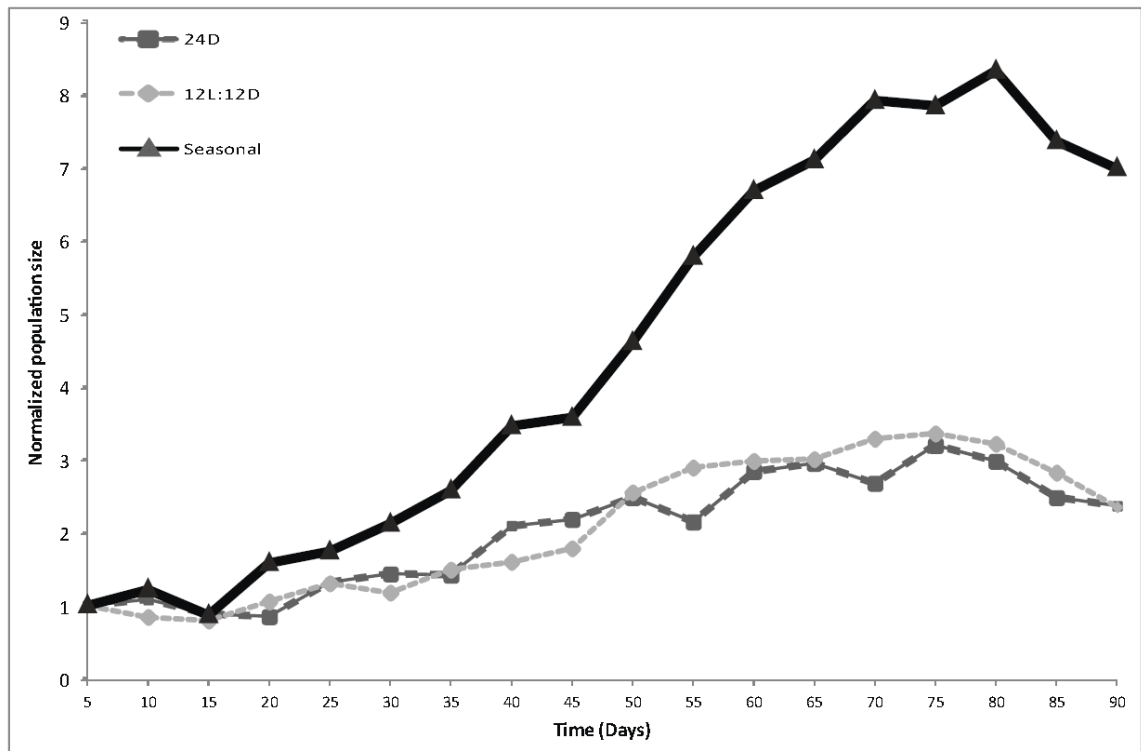


Figure 10