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ORIGINAL PAPER

Effects of protein-constrained brood food on honey bee (*Apis mellifera* L.) pollen foraging and colony growth

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Abstract Pollen is the sole source of protein for honey bees, most importantly used to rear young. Honey bees are adept at regulating pollen stores in the colonies based on the needs of the colony. Mechanisms for regulation of pollen foraging in honey bee are complex and remain controversial. In this study, we used a novel approach to test the two competing hypothesis of pollen foraging regulation. We manipulated nurse bee biosynthesis of brood food using a protease inhibitor that interferes with midgut protein digestion, significantly decreasing the amount of protein extractable from hypopharyngeal glands. Experimental colonies were given equal amounts of protease inhibitor-treated and untreated pollen. Colonies receiving protease inhibitor treatment had significantly lower hypopharyngeal gland protein content than controls. There was no significant difference in the ratio of pollen to nonpollen foragers between the treatments. Pollen load weights were also not significantly different between treatments. Our results supported the pollen foraging effort predictions generated from the direct independent effects of pollen on the regulation of pollen foraging and did not support the prediction that nurse bees regulate pollen foraging through amount of hypopharyngeal gland protein biosynthesis.

Keywords Honey bee · Pollen foraging · Protease inhibitor · Brood food · Hypopharyngeal glands

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Introduction

For the nonreproducing worker caste of social insect colonies, colony growth and reproduction are the principal sources of fitness. Honey bee colonies reproduce through a process of colony budding, commonly referred to as swarming (Winston 1987). In general, a larger adult population results in increased probabilities for colonies to reproduce and for swarms to survive; fitness traits (Cole 1984; Lee and Winston 1987; Little 1979; Michener 1964; Pomeroy 1979; Richards and Richards 1951; Seeley 1985a,b; Seeley and Visscher 1985; Winston 1987). The mechanism for colony growth is increased brood rearing. The honey bee (*Apis mellifera* L.), like most social insects, have a division of labor whereby individuals perform different tasks as they age. Brood rearing labor is divided among nurse bees and foragers. Ordinarily, younger nurse bees work within the nest directly tending larvae. Older bees are more probably found foraging for nectar or pollen outside the nest. Nurse bees consume forager-collected pollen to biosynthesize a proteinacious hypopharyngeal gland secretion called brood food that is progressively provisioned to larvae. Pollen is the only source of protein available to bees and it is through nurse bees that larvae are the principal consumers of protein in a colony. Pollen foragers collect pollen from flowers, carry it back to the colony on the outside of the body packed onto special structures of their hind legs called corbiculae, and directly deposit their loads into wax cells usually situated around brood rearing areas of the nest (Camazine 1991). The allocation of the foraging force profoundly affects colony growth and development (Farrar 1944; Moeller 1958, 1961; Free and Racey 1968; Nelson and Jay 1972; Smirl and Jay 1972; Pankiw et al. 2004). As such, the mechanisms that regulate the allocation of the pollen

foraging force are integral to an understanding of colony fitness.

Two hypothetical mechanisms dominate studies of pollen foraging regulation. The first is an "information center" model and subsequent modifications (Seeley 1985a,b, 1995; Seeley et al. 1991) named in this study as the brood food hypothesis. The second is a "stimulus–response threshold model", referred to in this study as the direct independent effects of stored pollen and brood (Page and Mitchell 1998; Page and Erber 2002; Scheiner et al. 2004).

The brood food hypothesis predicts that brood and stored pollen indirectly affect the behavior of pollen foragers through a single inhibitory signal (Camazine 1993; Seeley 1995). Bees are activated to collect pollen, thus regulation occurs through inhibition. With excess pollen stored in a colony there is also an excess of inhibitor that is presumably distributed to foragers by trophallaxis with nurse bees. If pollen is in surplus, it is hypothesized that nurse bees transfer more protein to foragers and inhibit pollen foraging. Brood food is the most likely inhibitor, thus the brood food hypothesis for the regulation of pollen foraging. Some information center based studies have focused on trophallaxis as mechanism for the transmission of information that may regulate pollen foraging (Camazine et al. 1998; Weidenmüller and Tautz 2002).

A competing hypothesis is that stored pollen and brood have direct, independent effects on pollen foraging. Many studies have demonstrated the effects of quantities of brood and stored pollen. Increasing the amount of larvae in colonies, or the chemical cues derived from larvae called brood pheromone, increases the number of pollen foragers and pollen load weights returned (Filmer 1932; Al-Tikrity et al. 1972; Free 1979; Eckert et al. 1994; Pankiw et al. 1998, 2004; Fewell and Bertram 1999; Pankiw and Page 2001; Pankiw and Rubink 2002; Pankiw 2004a–c. Pollen foraging activity level decreases in response to the addition of stored pollen (Free 1967; Barker 1971; Moeller 1972; Danka et al. 1987; Fewell and Winston 1992; Camazine 1993; Fewell and Bertram 1999) and increases in response to the removal of stored pollen (Free 1967; Fewell and Winston 1992; Camazine 1993). Increasing the amount of stored pollen in colonies concurrently increases brood rearing and decreases pollen foraging activity to a homeostatic set point (Fewell and Winston 1992). Empty comb space near the brood also stimulates pollen foraging behavior, while stored pollen clearly inhibits. Dreller et al. (1999) demonstrated that pollen foraging decreases only when foragers have direct access to stored pollen, and direct access to brood is necessary for an increase in pollen foraging response to an increase in amount of brood.

Using conventional colony-level manipulations is problematic because both the direct and indirect hypotheses predict the same pollen foraging outcomes. But for the different mechanistic reasons, both hypotheses predict pollen foraging decreases with additional quantities of stored pollen and pollen foraging increases with additional amounts of brood. Given a fixed amount of available comb area, there is an interaction between amount of stored pollen, number of larvae, and empty space. Changing one necessarily changes the others. However, manipulating amount of brood pheromone allows for a change in the perceived number of larvae without changing the allocation of comb area for larvae, pollen, and empty storage space (Pankiw et al. 1998; Pankiw 2004a–c; Le Conte et al. 2001; Pankiw and Rubink 2002). With brood pheromone added to colonies, the brood food hypothesis predicts no change in pollen foraging due to no change in demand for brood food. The direct independent effect of amount of brood and pollen hypothesis predicts an increase in amount of pollen foraging as a consequence of the increased pollen foraging stimulus of brood pheromone. Colonies treated with supplemental amounts of brood pheromone foraged more for pollen than did control colonies containing the same amount of brood and stored pollen (Pankiw et al. 1998, 2004; Pankiw and Rubink 2002; Schulz and Robinson 2002; Pankiw 2004a–c). Results of these studies support the direct, independent effects hypothesis, such that colonies approximated amount of larvae from their chemicals and foraged for pollen accordingly.

Direct experimental evidence of brood food protein as a feedback mechanism inhibiting pollen foraging has yet to be demonstrated. In this study, we manipulated nurse bee biosynthesis of brood food using a protease inhibitor that interferes with midgut protein digestion in adults, significantly decreasing the amount of protein extractable from hypopharyngeal glands (Sagili et al. 2005). Manipulating the amount of hypopharyngeal gland protein and controlling for amount of stored pollen resulted in the following predictions tested here: (1) the direct, independent effects hypothesis predicts no difference in pollen foraging effort because amount of stored pollen is the same in treated and control colonies vs (2) the brood food hypothesis predicts that protease inhibited colonies should allocate a greater pollen foraging effort due to a decreased amount of nurse produced protein. The primary objective in this experiment was to measure the effects of manipulating hypopharyngeal gland protein (brood food) content in nurse bees on pollen foraging.

Materials and methods

This experiment was replicated four times and had two treatments, 1% SBTI (soybean trypsin inhibitor) (Sigma-

Aldrich product T-9003, St. Louis, MO, USA) and control. Micronucleus hives made of styrofoam (25×19×14 cm) were used for this experiment and the experiment was conducted for a 30-day period. Each hive consisted of five frames, two frames pollen (476 cm^2), one frame honey (238 cm^2), 1/2 frame (119 cm^2) with brood and, 1 1/2 empty frames empty space (357 cm^2). Fifteen hundred newly emerged worker bees were introduced in to each hive and colonies were allowed to establish for 7 days. All the bees used in the experiment were obtained from a single colony source. Each experimental colony was headed by an unrelated queen. Control colonies received powdered pollen without SBTI packed into two frames, whereas SBTI-treated colonies received powdered pollen mixed with 1% SBTI (*w*:*w*) packed into two frames. After packing the pollen into the cells, the surface was sprayed with 50% sugar syrup (Dreller and Tarpy 2000). Each week, for a period of 4 weeks, 100 newly emerged bees from a common source were individually identified with a number tag glued to the thorax and released into each colony starting from initiation of the experiment. The brood consisted of 2-day-old eggs at the beginning of the experiment and abundant pollen was available in the environment during the entire experimental period.

Hypopharyngeal gland protein analysis

From each colony, on days 7 and 14, fifteen tagged bees from the brood nest area were collected for estimating hypopharyngeal gland protein content. Bees were cold euthanized, their hypopharyngeal glands dissected, and stored in Tris buffer at −80°C for further analysis. Protein content of hypopharyngeal glands was determined using Bradford assay as per Sagili et al. (2005). Briefly, hypopharyngeal glands were homogenized using a homogenizer that tightly fits in microcentrifuge tubes used to store the glands. Subsequently, tubes were centrifuged at 1,000 rpm for 2 min. Supernatant from each tube was used for analysis. We used the 500-0202 Quick Start Bradford Protein Assay Kit 2 (Bio-Rad Laboratories, CA, USA). We added 2 or 5 μl from each sample to be analyzed to microcentrifuge tubes containing 1 ml Bradford reagent. Tubes were vortexed to homogenize the contents, then incubated for 5 min at room temperature. Standard curves were prepared using bovine serum albumin (BSA). Protein absorbance was measured at 595 nm against blank reagent using a Beckman Spectrophotometer (Model #D4-640, Beckman Instruments, Columbia, MD, USA). Weight of protein (BSA) was plotted against the corresponding absorbance value to generate a linear regression equation (SAS PROC REG; SAS 2000). Protein extracted from hypopharyngeal glands was calculated using the linear regression equation generated above. Protein quantity was further analyzed using analysis of variance (ANOVA) (Sokal and Rohlf 1995; SPSS 2000).

Midgut proteolytic enzyme activity

The midguts were also excised from the same bees from which the hypopharyngeal glands were removed and midgut proteolytic enzyme activity was measured as per Sagili et al. (2005) briefly described below. Frozen guts were crushed, homogenized in Tris-HCl buffer (pH 7.9), and centrifuged at 10,000 rpm for 5 min. The supernatant was analyzed for total gut proteolytic enzyme activity (casenolytic activity) as described by Michaud et al. (1995). Five microliters of supernatant was used for each reaction. Twenty microliters of assay buffer (0.1 M Tris–HCl, pH 7.9) and 60 μl of 2% (*w*/*v*) azocasein diluted in assay buffer were added, respectively, to the supernatant and incubated for 6 h at 37°C. To remove the residual azocasein after proteolysis, 300 μl of 10%(*w*:*v*) TCA (Trichloro acetic acid) was added to each mixture and centrifuged for 5 min at 10,000 rpm. Three hundred fifty microliters of supernatant was added to 200 μl of 50% ethanol in water, and the absorbance of this mixture was measured at 440 nm using a Beckman DU 64 spectrophotometer. Total gut proteolytic activity was expressed in terms of OD_{440}. Mean total gut proteolytic enzyme activities from each treatment were analyzed using ANOVA (Sokal and Rohlf 1995; SPSS 2000).

Foraging behavior measurements

The number of foragers returning with visible pollen loads (pollen foragers) and those returning with no visible pollen load (nonpollen foragers) was counted for a 5-min period twice daily in the morning and afternoon beginning 24 h after pollen treatments were applied. Beginning on the fifth day, to the termination of the experiment, colony entrances were blocked with wire mesh for 15-min intervals between 0900 to 1600 hours for a total period of 2 h per day. Wire mesh was removed for a minimum of 30 min between each blocked interval.

Foragers with tags were captured individually in small cylindrical wire cages and their identity recorded. Pollen load weights of pollen foragers were measured by removing the pollen pellets from the corbicula of both the hind legs and weighing them. Age of first foraging was estimated by calculating the difference between the day of first observation as a returning forager and the day of emergence. Each week, the comb area occupied by eggs, larvae, pupae, pollen, and honey was measured using a metered grid (Pankiw et al. 2004).

Statistical analysis

Analysis of variance was used to analyze hypopharyngeal gland protein content, midgut enzyme activity, and pollen load weight. Correlation analysis (parametric) using SPSS was performed to measure the strength of linear association between midgut enzyme activity and hypopharyngeal gland protein quantity. Contingency table analysis was used to analyze the ratio of pollen to nonpollen foragers observed (Sokal and Rohlf 1995). Cox proportional hazards regression was used to analyze treatment effects on age of first foraging (PROC PHREG in Allison 1998; SAS 2000). Brood, pollen, and honey areas were analyzed using repeated-measures ANOVA.

Results

Hypopharyngeal gland protein content and midgut proteolytic enzyme activity

Hypopharyngeal gland protein content was significantly lower in bees treated with 1% SBTI vs the control for both 7- and 14-day-old bees (7 days, ANOVA $F_{1,118}=14.6$, $P<0.001$; 14 days, ANOVA $F_{1,118}=12.2$, $P<0.001$, respectively; Fig. 1). Midgut proteolytic enzyme activity was significantly lower in 1% SBTI-treated bees than control bees that were 7 and 14 days old (7 days, ANOVA $F_{1,118}=167.5$, $P<0.0001$; 14 days, ANOVA $F_{1,118}=139.5$, $P<0.0001$; Fig. 2). Midgut enzyme activity explained 29.3% of the variation in hypopharyngeal gland protein quantity (correlation analysis $\rho=0.293$, $P=0.01$).

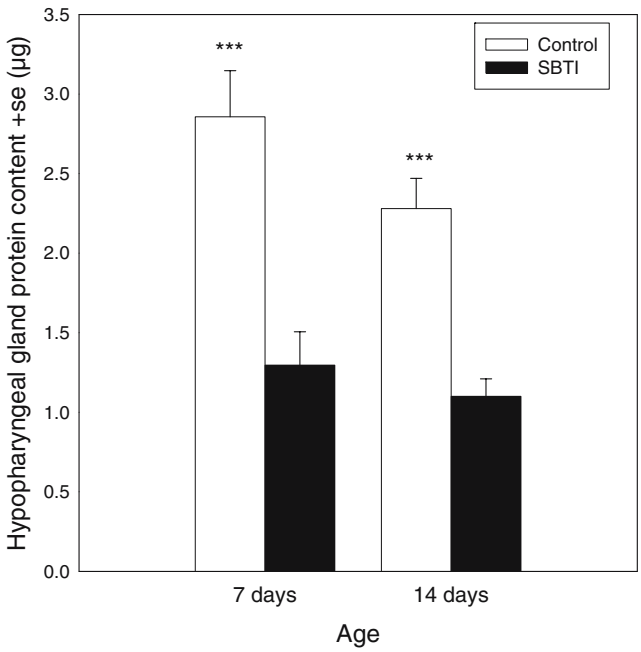

Fig. 1 Mean hypopharyngeal gland protein quantities of bees (+SE) that received control and SBTI treatments. *Asterisk* indicates significant difference ($P<0.001$). For statistical details, see text

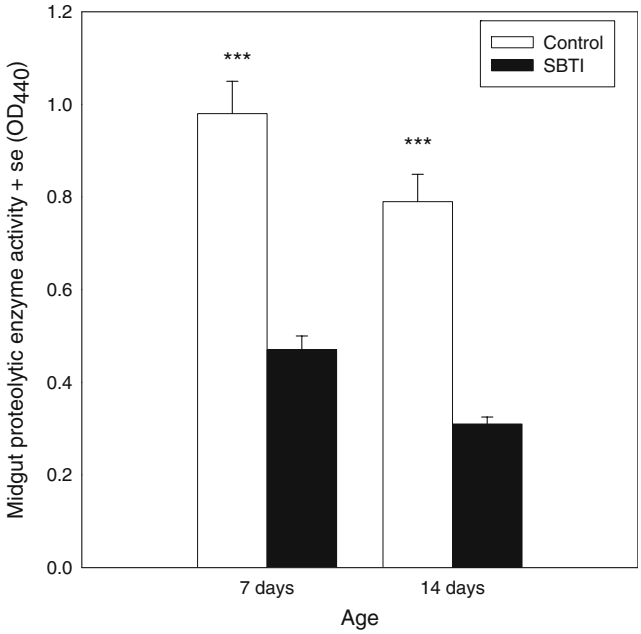

Fig. 2 Mean midgut proteolytic enzyme activities (+SE) of bees that received control and SBTI treatments. *Asterisk* indicates significant difference ($P<0.0001$). For statistical details, see text

Foraging behavior

There was no significant difference in the ratio of pollen to nonpollen foragers entering the colonies both in the morning and afternoon (morning: $\chi^2=18.3$, 1 *df*, $P=0.63$; afternoon: $\chi^2=16.9$, 1 *df*, $P=0.34$; Fig. 3) between SBTI treatments and controls. Number of pollen foragers evaluated for control and SBTI treatments were 1,765 and 1,708, respectively, and the number of nonpollen foragers evaluated were 6,070 and 5,594, respectively, for control and SBTI treatments. Pollen load weight was not significantly different between the 1% SBTI treatment and control ($F_{1,6}=1.9$, $P=0.45$; Fig. 4). Number of pollen foragers evaluated for pollen load weight analysis were 600 each for the control and SBTI treatments. Bees that received 1% SBTI treatment foraged at a significantly younger mean age than the control bees ($\chi^2=9.3$, $P<0.01$, $e^{\beta}=0.67$). The hazard ratio statistic, e^{β}, was transformed to a more meaningful statistic indicating that bees ingesting 1% SBTI were 33% more at risk to forage than control bees over the 30-day experimental period (Allison 1998). Mean age of first foraging in SBTI treatments and controls was 11.3±0.4 (SE) days and16.2±0.7 (SE) days, respectively.

Colonies that received SBTI treatment reared significantly less brood area than control colonies (repeated measures $F_{1,6}=14$, $P=0.003$; Fig. 5). Pollen and honey areas were not significantly different between the SBTI

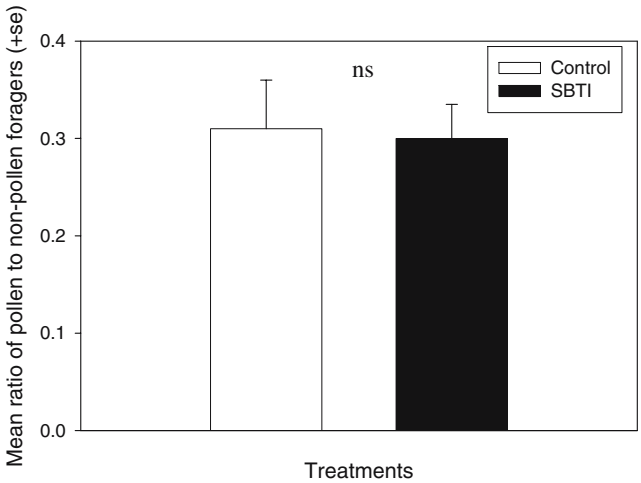

Fig. 3 Mean ratio of pollen to nonpollen foragers (+SE) entering the colonies ($P>0.05$). For statistical details, see text. *ns* Indicates no significant difference. Pollen foragers: $n=1{,}765$ (control) and $n=1{,}708$ (SBTI). Nonpollen foragers: $n=6{,}070$ (control) and $n=5{,}594$ (SBTI)

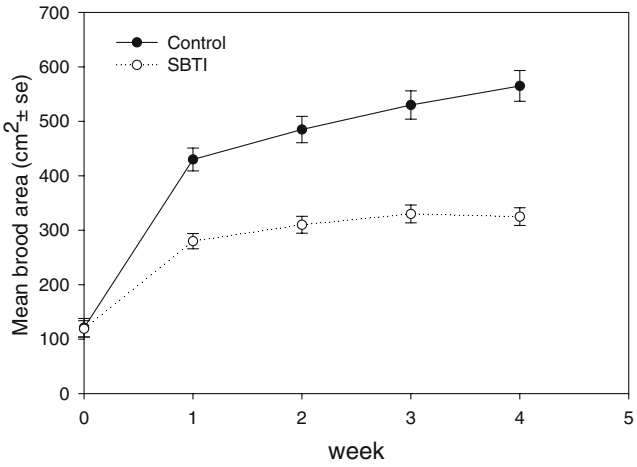

Fig. 5 Mean brood area reared (±SE) in control and SBTI treated colonies over a period of 4 weeks

treatment and control colonies (repeated measures $F_{1,6}=1.4$, $P=0.1$ and $F_{1,6}=0.9$, $P=0.35$, respectively). There was no significant difference in the mortality between 1% SBTI treatments and controls ($F_{1,6}=4.8$, $P=0.12$). The mean number of adult bees surviving at the termination of the experiment in SBTI treatments and controls were 1,585± 8.89 (SE) and 1,634.5±20.66 (SE), respectively.

Discussion

The results of this study supported the prediction of no difference in pollen foraging effort between SBTI-treated vs control colonies generated by the direct, independent effects hypothesis. Equivalent amounts of stored pollen in SBTI and control colonies were maintained throughout the course of the experiment. The ratio of pollen foragers and weight of pollen loads returned were similar between treatments. Amount of protein extractable from the hypopharyngeal glands of bees reared in SBTI-treated colonies was significantly lower than those reared in control colonies. Hypopharyngeal glands in bees fed 0.1 or 1% SBTI (*w*:*v* in sucrose solution) are lighter in weight and have smaller acini when compared to controls (Babendreier et al. 2005). In this experimental paradigm, foragers appeared to be assessing need for pollen based on amount of stored pollen in colonies rather than by amount of hypopharyngeal gland protein extractable from workers.

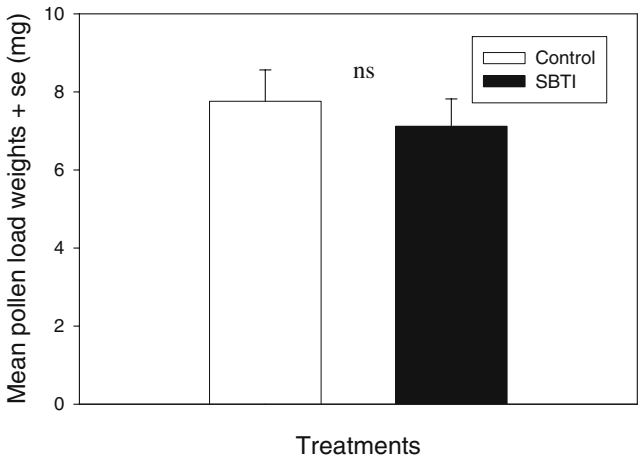

Fig. 4 Mean pollen load weights (+SE) collected by control and SBTI fed colonies ($P>0.05$). For statistical details, see text. *ns* Indicates no significant difference

The protease inhibitor used in this study inhibited midgut proteolytic enzyme activity of workers. Interference with midgut protein digestion was strongly associated with decreased amounts of protein extractable from the hypopharyngeal glands of colony-reared workers feeding on a pollen diet containing SBTI as well as caged-workers reared in an incubator (Sagili et al. 2005). Similar results were reported by Burgess et al. (1996), where bees that fed the highest dose of SBTI (1%) had significantly lower levels of three endopeptidases, chymotrypsin, elastase, and trypsin. An inference of these physiological results is that bees ingesting SBTI were poor producers of brood food. This conclusion is supported by the significantly lower amount of brood area reared by SBTI vs control colonies. Lower amount of brood can be attributed to poor nutritional environment in the colonies resulting from decreased hypopharyngeal gland protein. It is important to note that despite the greater amount of brood area reared by control colonies, measures of pollen foraging remained statistically similar between treatments. This is further support for the direct, independent effects hypothesis such that there is a response threshold for amount of brood area at or beyond which increased pollen foraging is released (Page and

Mitchell 1998). Although there was more brood in control colonies, it was not sufficiently great to induce more pollen foraging. This is consistent with results where brood pheromone amount is increased incrementally to a point where increased pollen foraging is observed (Pankiw et al. 1998; Pankiw and Page 2001).

SBTI interference of protein digestion in adults was associated with decreased age of first foraging. This could be interpreted as a "stressor" effect of SBTI on adult length of life. In general, foraging behavior is the terminus on the honey bee behavioral ontogenetic pathway (Winston 1987). Some factors associated with decreased age of first foraging may be viewed as stressors. For example, the handling of newly emerged adults (Pankiw 2003), removal of the foraging caste from colonies (Huang and Robinson 1992), exposure to primer pheromones (Le Conte et al. 2001; Pankiw 2004a,b), and mite infection (Korpela et al. 1992). Schulz et al. (1998) reported that shortage of food in honey bee colonies accelerated behavioral development, and starved colonies had significantly greater proportions of precocious foragers. Malone et al. (2001) demonstrated that bees fed aprotinin, a serine protease inhibitor, started flying 2.8 days earlier than control bees. Pollen and honey areas of control and SBTI treatments were identical which suggests that pollen and nectar foraging were similar in both the treatments. At the termination of the experiment SBTI, treatments had fewer bees compared to the controls, but this difference was not significant. This suggests that the SBTI treatment did not adversely effect the survival of the bees. It is possible larvae were directly affected by the protease inhibitor, but only in the later stages, when they are fed small quantities of pollen along with the hypopharyngeal gland and mandibular gland secretions. However, this effect is not expected to be significant. In honey bee larvae, pollen constitutes only a minor part of the protein supply (Babendreier et al. 2004).

Multiple methods have now been utilized to attempt to generate predictions that clearly point to specific mechanisms for the regulation of pollen foraging. To date, the majority of studies support the direct, independent effects hypothesis. However, despite all efforts, the question of how honey bee colonies regulate pollen foraging remains controversial. Models of behavioral organization in social insects all predict that workers will vary task performance in response to common environmental cues (Seeley 1985a,b; Tofts and Franks 1992). The specific hypotheses addressed in these studies were developed to address different foraging behaviors; the indirect hypothesis was originally developed to address nectar foraging and the direct hypothesis addressed pollen foraging. They sometimes make different and competing assumptions about what produces variation in individual responses to stimuli and how individuals receive information about the colony environment that changes foraging responses.

Pitting one hypothesis against the other has been largely intractable, making hypothesis falsification impossible or unresolved because they generate the same predictions but for different reasons, or a model is modified to address an unexpected result. A philosophical resolution may be to adopt "integrative pluralism" (Mitchell 2002). Integrative pluralism recognizes that complex systems may comprise multiple causes. Theories and explanations are not always competing (Sherman 1988). Integrative pluralism allows for models working at the same level of analysis to be combined for a more complete synthesis. Fewell and Bertram (1999) generated predictions from central information and threshold models for honey bee-foraging behavior responses to gradual increases in amount of stored pollen. Although not demonstrated directly, their results suggested that the regulation mechanisms forwarded by both hypotheses may be operating concurrently and they proposed a model that integrated the two mechanisms. Thus, there is recognition that factors identified through tests of models of colony organization may be at work concurrently or hierarchically. Integrative pluralism may be the next more fruitful direction to pursue insights to what is clearly a complex system.

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