# Brood Pheromone Effects on Colony Protein Supplement Consumption and Growth in the Honey Bee (Hymenoptera: Apidae) in a Subtropical Winter Climate

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**ABSTRACT** Fatty acid esters extractable from the surface of honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), larvae, called brood pheromone, significantly increase rate of colony growth in the spring and summer when flowering plant pollen is available in the foraging environment. Increased colony growth rate occurs as a consequence of increased pollen intake through mechanisms such as increasing number of pollen foragers and pollen load weights returned. Here, we tested the hypothesis that addition of brood pheromone during the winter pollen dearth period of a humid subtropical climate increases rate of colony growth in colonies provisioned with a protein supplement. Experiments were conducted in late winter (9 February–9 March 2004) and mid-winter (19 January–8 February 2005). In both years, increased brood area, number of bees, and amount of protein supplement consumption were significantly greater in colonies receiving daily treatments of brood pheromone versus control colonies. Amount of extractable protein from hypopharyngeal glands measured in 2005 was significantly greater in bees from pheromone-treated colonies. These results suggest that brood pheromone may be used as a tool to stimulate colony growth in the southern subtropical areas of the United States where the package bee industry is centered and a large proportion of migratory colonies are overwintered.

**KEY WORDS** honey bee, brood pheromone, winter colony growth

In the United States, many honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), colonies are overwintered in the warmer southern states, mainly to increase the probability of winter survival. For example, the number of colonies in Texas increases by >100% from November to February (Texas State Entomologist 2004). Migratory colonies are moved out of the state from early February to mid-March, mainly to pollinate almonds in California, and then to honey-producing areas. In addition to providing a warm winter refuge for migratory colonies, the southern states are particularly important sources of package bee (replacement/expansion) colonies. Typically, a package of bees consists of 1.4 kg of young adult workers harvested from parent colonies that are shipped to buyers with or without a queen as requested. A sufficient number of bees must be retained in the parent colony to ensure its survival and future productivity. Therefore, the number of bees harvested for packages increases with the size of the parent colony. Package bee shipments begin in late March and may extend to early June (unpublished data). One characteristic in most areas of a subtropical Texas winter is a dearth of flowering plant pollen, the sole source of protein for honey bees. From December to February, nonflowering Cupressaceae pollen is most abundant in the region, and it is not collected by bees (Garrett 2002). Another characteristic of the humid subtropical winter is frequent rains that also prevent foraging. Consequently, colonies rear only small amounts of brood by using pollen stores that were collected in the previous season. In this way, colony growth is constrained in a subtropical winter in part by the availability of pollen.

In general, foragers respond to changes in foraging environment such that when food resources are abundant, foraging increases; and when food resources are scarce, foraging decreases or ceases, depending on amount of feedback stimulus (von Frisch 1967). Pollen foraging is also dependent on intracolony stimuli, such that pollen foraging decreases with the addition of stored pollen or removal of larvae from colonies (Free 1967, Danka et al. 1987, Fewell and Winston 1992, Camazine 1993). Conversely, pollen foraging increases in response to the removal of stored pollen from colonies, and increased amount of larvae in colonies (Free 1967, Al-Tikrity et al. 1972, Free 1979, Fewell and Winston 1992, Camazine 1993, Eckert et al. 1994, Pankiw et al. 1998, Pankiw and Page 2001). Pollen foragers collect pollen from floral sources when available, fly back to the nest and deposit their loads of pollen directly into wax comb cells. Stored pollen

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is consumed by nurse bees that convert pollen-derived proteins into hypopharyngeal gland secretions that are progressively provisioned to developing larvae (Crailsheim et al. 1992). Thus, it is through nurses that larvae are the primary consumers of protein in the colony.

Chemical cues on the surface of larvae called brood pheromone have multiple effects on individual and colony-level pollen foraging, as well as physiological effects on hypopharyngeal glands of adults collected from the brood nest. Adding brood pheromone to colonies increases the number of pollen foragers by up to 150%, significantly increases pollen load weight returned by individual pollen foragers and the number of pollen grains extractable from the bodies of non-pollen foragers, increases the number of pollen forager trips per unit time, significantly decreases the age workers begin to forage, and significantly increases colony growth rate in the spring and summer (Pankiw et al. 1998; Le Conte et al. 2001; Pankiw and Page 2001; Pankiw and Rubink 2002; Pankiw 2004a,b, 2007; Pankiw et al. 2004). Bees reared in a brood pheromone supplemented cage or colony environment have significantly greater amounts of protein extractable from their hypopharyngeal glands compared with those without pheromone supplement (Mohammedi et al. 1996, Pankiw et al. 2004).

Beekeepers commonly provide a protein/pollen supplement to colonies during periods of low pollen availability to stimulate colony growth (Waller et al. 1981, Herbers 1992, Nabors 2000, Saffari et al. 2004, van der Steen 2007). However, because larvae may be absent or at their lowest levels depending on climate in the winter or early spring (Winston 1987), there is very little if any larval stimulus to induce consumption of protein supplements (Hrassnigg and Crailsheim 1998a,b). Additionally, low to no brood pheromone results in low or no hypopharyngeal gland development and protein biosynthesis in adult bees (Mohammedi et al. 1996). In a temperate climate, winter brood rearing ceases, and hypopharyngeal glands are reduced in size and have low rates of protein synthesis along with low amounts of extractable protein (Brouwers 1983). Little to nothing has been reported on hypopharyngeal gland protein amount and growth in colonies overwintering in a U.S. subtropical winter. Here, we tested the hypothesis that addition of synthetic brood pheromone to colonies during mid- and late winter of a humid subtropical climate with a characteristic pollen dearth stimulates protein supplement consumption, amount of brood and bees reared, and hypopharyngeal gland protein content.

## Materials and Methods

**Experiment 1 (2004).** The objective of this experiment was to compare the effect of brood pheromone on honey bee colony growth during late winter pollen dearth in a humid subtropical region of Texas. This period of time was approximately that period of time package bee producers typically feed colonies to stimulate increased young adult bee production. The experiment was conducted from 9 February to 9 March 2004 in a College Station, TX, apiary (30° 6′ N; 96° 32′ W; classified as a humid subtropical climate). The daily average high temperature was 17°C, and the average low temperature was 8°C over the course of the experiment. Twelve (12) honey bee colonies from Texas A&M University apiaries were randomly selected for the experiment. One day before treatment colony measures were estimated using a grid the size of a Langstroth-deep frame divided into 6.45-$cm^2$ sections. The area covered by bees was converted to bee numbers by a factor of 1.5 bees per $cm^2$ (Pankiw et al. 2004). Comb area occupied by brood (eggs, larvae, and pupae), honey, pollen, and empty space also was measured. Subsequently, bee number estimates and brood area measures were conducted every 7 d during the course of the experiment. Ten days after terminating, the experiment numbers of adult bees were once more estimated because this time was sufficient for eggs laid in the first week of the experiment to emerge as adults. Six colonies were randomly selected to each receive brood pheromone at 1.12 mg/d (Pankiw et al. 1998, 2004; Pankiw and Rubink 2002; Pankiw 2004a,b, 2007). Brood pheromone is comprised of 10 fatty acid esters here formulated as follows: 1% ethyl linoleate, 13% ethyl linolenate, 8% ethyl oleate, 3% ethyl palmitate, 7% ethyl stearate, 2% methyl linoleate, 21% methyl linolenate, 25% methyl oleate, 3% methyl palmitate, and 17% methyl stearate. The pheromone was dissolved in 1 ml of HPLC grade 2-propanol (Sigma-Aldrich, St. Louis, MO) that was equally distributed on two sides of a 328-$cm^2$ glass plate previously rinsed with solvent. Six (6) colonies each received a daily control treatment consisting of one glass plate also rinsed with 2-propanol. The 2-propanol was completely evaporated before suspending the glass plate in the middle of the brood nest area of each colony.

Each colony was provisioned with a commercially available protein supplement: 450 g of Brood Builder (Dadant & Sons, Hamilton, IL), moistened with 50 ml of a 30% sucrose solution. The protein supplement was then flattened into an ≈1-cm-thick patty between two pieces of wax paper that was later scored before placement. Each colony received one patty placed on the top bars of the brood nest area. Every 7 d, protein supplement patties were removed, weighed, and replaced with a fresh patty, regardless of amount remaining. At no time were colonies without a protein supplement patty. Given that time of year represented late winter in College Station, TX, colony entrances were monitored every 3 d for a 5-min interval for incoming pollen foragers, or whenever it was not raining. Pollen foragers were observed entering colonies on 9 March 2004, triggering termination of the experiment.

**Experiment 2 (2005).** The objective of this experiment was to compare the effect of brood pheromone versus nonpheromone control treatment on honey bee colony growth during the mid-winter pollen dearth. Typically, preparations for meeting pollination contract standards for amount of brood area and bees

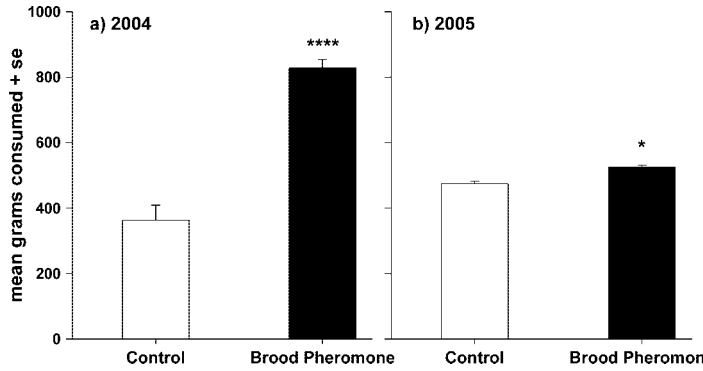


**Fig. 1.** Consumption of protein supplement by control and brood pheromone treated colonies in 2004 (a) and 2005 (b) (****, $P < 0.0001$; *, $P < 0.05$).

are being made at this time of year (Traynor 1993). The experiment was conducted from 19 January to 8 February 2005 in the same apiary as in 2004 but in different colonies. The daily average high temperature was 16°C, and the average low was 6°C. The protocol was the same as experiment 1, except each treatment was replicated eight times at the colony level. As in 2004, numbers of adult bees were estimated 10 days after treatments ceased. In this year, we additionally tested the hypothesis that brood pheromone affects amount of hypopharyngeal gland protein of winter bees in January and February. Every week for 3 wk, 10 bees were collected from the brood nest area of each colony for measurement of hypopharyngeal gland protein by using the Bradford Assay (Sagili et al. 2005, Sagili and Pankiw 2007).

Bees were cold euthanized, and then their hypopharyngeal glands dissected. The glands were stored in Tris buffer at −80°C before further processing. Hypopharyngeal glands were macerated in microcentrifuge tubes by using a small plastic pestle and vortexed to homogenize the solution. Subsequently, the homogenate was centrifuged at 1,000 × *g* for 2 min. Supernatant from each tube was used for protein analysis. We used the 500-0202 Quick Start Bradford protein assay kit two (Bio-Rad Laboratories, Hercules, CA). Then, 2 and 5 μl were added from each sample to be analyzed in microcentrifuge tubes containing 1 ml of Bradford reagent. Tubes were vortexed, and then they were 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 spectrophotometer (model D4–640, Beckman Instruments, Inc., Columbia, MD). Weight of protein (BSA) was plotted against the corresponding absorbance value to generate a linear regression equation (SPSS Inc. 2005). Protein extracted from hypopharyngeal glands was estimated using the linear regression equation generated above. Bees were handled in compliance with current laws of the United States of America.

## Results

**Experiment 1 (9 February–9 March 2004).** At the time of initiation, on average, the colonies used in this experiment consisted of ≈12,000 adult bees (approximately six completely covered Langroth-deep frames), 2,600-cm$^2$ brood area (approximately two frames), and one mated queen. Hives were made up of two Langstroth-deep supers, with a total of 10 frames. Amount of brood area was not significantly different between treatments (analysis of variance [ANOVA]: $F_{1,10} = 3.0$; $P = 0.1$), and, in particular, larval areas were not significantly different (brood pheromone, 326.3 ± 115.6 cm$^2$; control, 370.5 ± 122.5 cm$^2$; ANOVA: $F_{1,10} = 0.70$; $P > 0.05$). Amount of bees (ANOVA: $F_{1,10} = 1.2$; $P > 0.05$), honey area (ANOVA: $F_{1,10} = 3.3$; $P = 0.1$), pollen area (ANOVA: $F_{1,10} = 1.0$; $P > 0.05$), and empty space (ANOVA: $F_{1,10} = 4.1$; $P > 0.05$) in colonies randomly selected for pheromone or control treatments also were not significantly different.

Overall, colonies treated with brood pheromone consumed significantly more protein supplement than control colonies (ANOVA: $F_{1,10} = 80.0$; $P < 0.0001$) (Fig. 1a). There were significant differences between weeks for supplement consumption ($F_{2,10} = 20.0$; $P < 0.0001$), but no significant week × treatment interaction ($F_{2,10} = 1.1$; $P > 0.05$), meaning that treatment did not differentially affect supplement consumption from week to week. Repeated measures Mauchly's test indicated that the assumption of sphericity was violated for change in brood area data [$\chi^2(2) = 12.5$, $P < 0.05$]; therefore, degrees of freedom were corrected using Greenhouse–Geisser estimates of sphericity ($\varepsilon = 0.571$); a default correction in SPSS 14.0 (SPSS Inc. 2005). There was a significant effect of week on change in brood area (ANOVA: $F_{1.1,11.4} = 5.2$; $P < 0.05$) such that there was a significant amount of increased brood area in pheromone-treated colonies versus control colonies, in weeks 1 and 2 of the experiment (Fig. 2a). There was a significant interaction of week × treatment on amount of change in brood area (ANOVA: $F_{1.1,11.4} = 7.4$; $P < 0.05$), an indication that amount of change in brood area was not consistent between treatments from week to week (Fig. 2a).

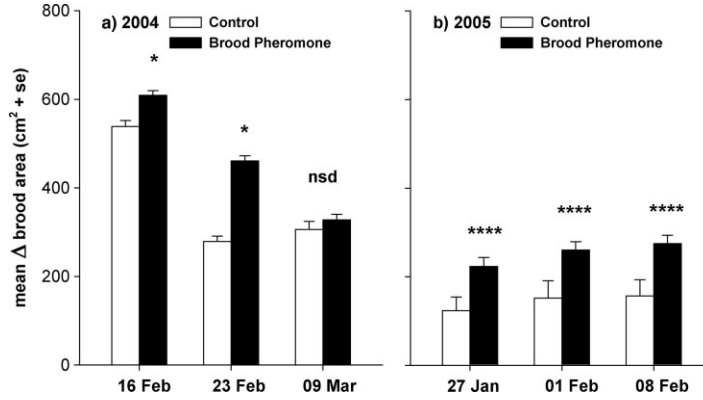


**Fig. 2.** Amount of change in brood area in control and brood pheromone-treated colonies in 2004 (a) and 2005 (b) (nsd, not significantly different; *, $P < 0.05$; ****, $P < 0.000$).

Brood pheromone treatment had a significantly positive effect on the mean increase in the estimated number of adult bees compared with control colonies (ANOVA: $F_{1,10} = 37.0$; $P < 0.001$) (Fig. 3a).

**Experiment 2 (19 January–8 February 2005).** At the outset, colonies used in the experiment contained one mated queen, approximately six frames fully covered by adults (≈12,000), and approximately two frames of brood (≈2,600 $cm^2$). Hive size was the same as in 2004. At the onset of the experiment, brood area was not significantly different between treatments (ANOVA: $F_{1,14} = 0.8$; $P > 0.05$), including larva area (brood pheromone, 357.5 ± 117.3 $cm^2$; control, 368.4 ± 120.1 $cm^2$; ANOVA: $F_{1,14} = 0.05$; $P > 0.05$). Additional colony measures were also not significantly different between pheromone and control colonies for amount of bees (ANOVA: $F_{1,14} = 0.03$, $P > 0.05$), honey area (ANOVA: $F_{1,14} = 1.0$; $P > 0.05$), pollen area (ANOVA: $F_{1,14} = 1.2$; $P > 0.05$), and empty space (ANOVA: $F_{1,14} = 3.0$; $P > 0.05$). Total consumption of protein supplement was significantly different between pheromone treated and control colonies (ANOVA: $F_{1,14} = 5.1$; $P < 0.05$) (Fig. 1b). Colonies treated with brood pheromone reared significantly greater areas of brood than control colonies during the course of the experimental period (ANOVA: $F_{1,14} = 173.0$; $P < 0.0001$) (Fig. 2b). There was a significant effect of week on amount of change in brood area (repeated measures ANOVA: $F_{2,28} = 3.7$; $P < 0.05$), signifying amount of change brood area was significant from week to week (Fig. 2b). However, there was no significant interaction of treatment × week (repeated measures ANOVA: $F_{2,28} = 0.2$; $P > 0.05$), meaning that amount of change in brood area was consistent between treatments from week to week (Fig. 2b). Increase in number of adult bees was significantly greater in brood pheromone-treated colonies versus control colonies at the end of the experiment period (ANOVA: $F_{1,14} = 35.0$; $P < 0.0001$) (Fig. 3b). Amount of protein extractable from the hypopharyngeal glands of bees in brood pheromone-treated colonies was significantly greater than for control colonies (repeated measures ANOVA: $F_{2,28} = 9.1$; $P < 0.001$) (Fig. 4).

## Discussion

Overall, daily applications of brood pheromone to colonies during mid- and late winter in College Station, TX, significantly increased amount of protein supplement consumption and increased amount of

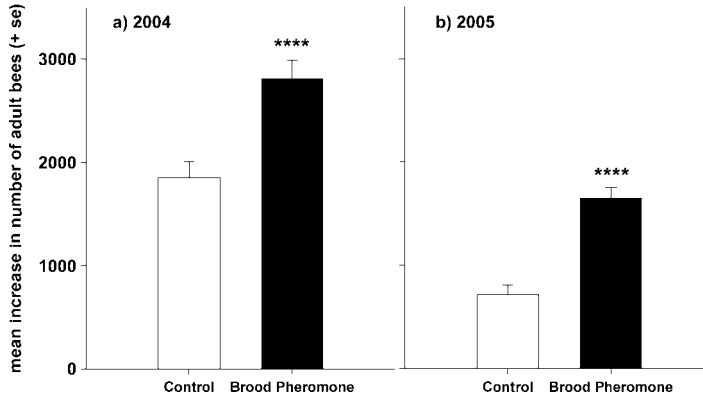


**Fig. 3.** Amount of change in adult bee area from day 1 to 31 in control and brood pheromone-treated colonies in 2004 (a) and 2005 (b) (****, $P < 0.000$).

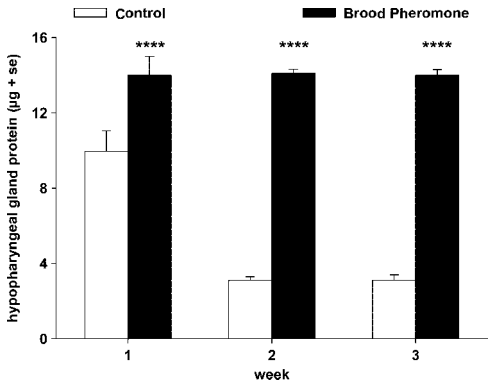


**Fig. 4.** Amount of protein extractable from hypopharyngeal glands of bees reared in control and brood pheromone-treated colonies in 2005 (****, $P < 0.000$).

bees and brood area in colonies. Additionally, in 2005 bees from pheromone-treated colonies showed an increased amount of protein extractable from the hypopharyngeal glands. Increased colony growth as a consequence of long-term brood pheromone treatment was observed previously in spring (Pankiw et al. 2004) and summer colonies (R.R.S. and T.P., unpublished data); however, it was not clear whether rate of growth could be significantly stimulated during a humid subtropical winter. The results of this study support the hypothesis that brood pheromone stimulates protein supplement consumption and colony growth, strongly suggesting that brood pheromone may be used as an apicultural tool to increase rate of colony growth during the winter in a humid subtropical climate.

The most apparent mechanism through which brood pheromone seemed to increase colony growth was through an increased larval food protein environment facilitated by increased protein supplement consumption and amount of protein extractable from hypopharyngeal glands. In the spring, summer, and fall when pollen is available, brood pheromone increases amount of pollen intake, but the amount of stored pollen does not change (Pankiw et al. 1998, 2004; Pankiw and Page 2001; R.R.S. and T.P., unpublished data). We have speculated that additional incoming pollen is rapidly consumed by nurses. The results of this study, although not directly comparable, lend strong support to the hypothesis that brood pheromone stimulates consumption of protein. A cascade of changed behaviors has been observed as a consequence of brood pheromone induced increased protein consumption. We have also shown that amount of time spent feeding the queen in brood pheromone-treated colonies is greater than control colonies (R.R.S. and T.P., unpublished data). Queen behaviors also are changed with brood pheromone treatment, such as significantly increased number of eggs laid, decreased idle time, and increased amount of time patrolling, presumably to locate cells for egg deposition (R.R.S. and T.P., unpublished data).

Colony sizes were not different between years; however, consumption of protein supplement and rate of growth were significantly greater in 2004. Overall, average high and low temperatures were similar in both years; therefore, temperature alone does not sufficiently explain differences. However, time of winter may have been an important factor such that brood pheromone treatments began 3 wk later in 2004. In 2004, colonies were experiencing ≈45 min more daylight than 2005 colonies during the experimental period (National Oceanic and Atmospheric Administration). Individual bees have been demonstrated to show age-related changes in circadian rhythm in spring and summer (reviewed in Elekonich and Roberts 2005). Although only speculative, the coevolutionary history of flowering plants and honey bees favors integrated seasonal photoperiodicity. A reasonable hypothesis is that honey bee colonies respond to seasonal photoperiodic cues associated with increased brood rearing stimulating increased protein supplement consumption.

Application of brood pheromone in both mid- and late winter significantly increased amount of brood rearing in colonies. However, in 2004 applications began 9 February where significant differences between treatments were observed during the first 2 wk but not the third week. In contrast, 2005 applications began on 19 January, and amount of colony growth was consistently significantly greater from week to week in pheromone-treated colonies. However, the amount of increase in brood area from week to week was greater in 2004 compared with 2005. Again, time of winter is the most likely explanation for observed differences in amount of colony growth between years.

Larvae were present in control colonies as stimulants for the consumption of protein supplement and hypopharyngeal gland protein biosynthesis. Curiously, in 2005 amount of extractable protein in control colonies was significantly lower in weeks 2 and 3 compared with week 1 ($P < 0.0001$) (Fig. 4). A similar drop in protein content was not observed in brood pheromone-treated colonies where amount of extractable protein remained consistently high from week to week (Fig. 4). Almost nothing is known of the physiological status of adult honey bees overwintering in the subtropical United States. It is possible we observed a more flexible intermediate state between the classical temperate winter bee and a summer bee that responded to unknown factors in control colonies and more clearly to brood pheromone. Most of what is known of winter bee behaviors and physiology is based on research performed in temperate climates with cold winters, such as in The Netherlands (Brouwers 1983), Switzerland (Fluri et al. 1977), Germany (see citations in Brouwers 1983), and Canada (Mattila and Otis 2007). Given that much of the queen breeding, package bee industry, and thousands of overwintering migratory colonies are located in the southeastern subtropical United States highlights a significant gap in our basic understanding of the physiology and states of colonies in which there is no cessation of brood rearing. It also would be interesting to examine

seasonal effects of brood pheromone supplementation on colonies in a temperate climate in which brood rearing ceases.

Stimulating colony growth with brood pheromone also increases the probability of colony-level reproduction called swarming. Natural swarming is viewed negatively by beekeepers because a swarm that issues from the nest is usually lost and what remains is a colony that is about half its original size and consequently less productive. However, beekeepers also take advantage of honey bee colony growth leading to reproduction as a means to increase colony numbers; replace dead colonies; and especially in the southeast, to sell package bees to other beekeepers. Instead of allowing colonies to swarm naturally, beekeepers choose the time of reproduction by manually dividing a large colony into two to three additional units, depending on parental colony size. To each divide, a mated queen is introduced, resulting in new colonies. Frequency of colony division and number of divisions are dependent on rate of colony growth and size of parental colony. The new colonies may be added to the beekeeper's apiary or sold to other beekeepers. In this respect, the addition of brood pheromone in the winter may be suited to a number of apicultural applications, such as increasing colony size for honey production, crop pollination, and bee production for the package bee industry. What remains to be investigated are possible trade-offs associated with stimulating growth, such as effects on *Varroa* mite populations, larval diseases, and swarming management.

The brood pheromone application delivery method used in this experiment as well as the cost of reagent grade chemicals is not practical for apiculture. We have recently developed a long-term, slow release method and an economical pheromone formulation of equal bioactivity to that of the reagent grade formulation, reducing the overall cost of brood pheromone treatment by >95% (T.P., J. P. Lafontaine, N. Avelino, A. L. Birmingham, and J. H. Borden, unpublished data). The availability of this new pheromone technology (SuperBoost; Phero Tech International Inc., Delta, BC, Canada) promises to add a new colony management tool for beekeepers and growers of bee pollinated crops, as well as a providing a tool for honey bee researchers.

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