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Effects of essential amino acid supplementation to promote honey bee gland and muscle development in cages and colonies

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ABSTRACT

There is growing concern about the impact of poor nutrition on honey bee health. With caged bee experiments and whole-colony field experiments, we examined the effects of supplementing bees with essential amino acids (EAA), or a control treatment of nonessential amino acids (NAA). Caged bees fed EAA developed significantly greater head weights than controls, weights that were similar to nurse bees. Caged bees fed EAA developed significantly greater thorax weights than controls, weights that were similar to foragers. Higher head and thorax weights may respectively reflect increased glandular development in nurse bees and higher flight muscle mass in forager bees. In our field study, 29% of the pollen collected by our honey bee colonies came from eucalyptus trees. Amino acid analyses revealed no EAA deficiencies for the bee-collected polyfloral pollen or for monofloral eucalyptus pollen. Colonies fed 29 g EAA supplement may have slightly increased individual bee growth and brood rearing, but this effect was not significant. A clear colony result was a correlation between nurse bee physiology and brood development: 17% increase in nurse bee weight corresponded to 100% more capped brood cells ($R^2 = 0.38$). We suggest that colony supplementation should target nurse bee nutrition. Nurse bees eventually become forager bees. Hence, increased glandular development may support colony brood development and greater flight muscle mass may assist colony foraging.

1. Introduction

Globally, honey bees (*Apis mellifera*) provide economically valuable agricultural pollination and are ecologically important pollinators (Hung et al., 2018). Agricultural intensification is, however, degrading the carrying capacity of agro-ecosystems for beneficial insect communities (Hallmann et al., 2017). Bee health decline has been directly attributed to interactions between nutritional stress, parasites, and pathogens (Dolezal and Toth, 2018). Additionally, poor nutrition amplifies pesticide toxicity in honey bees (Tosi et al., 2017), contributing to a complex pattern of synergistic threats.

Impaired nutrition arising from reduced forage is a major concern for securing pollinator populations. For honey bee colonies, proper nutrition is crucial for long-term health and survival (Brodschneider & Crailsheim 2010; Avni et al., 2014; Wright et al., 2018). Insufficient nutrition may cause a gradual decline of workers, which reduces colony fitness. Supplementary feeding has proven valuable for overcoming periods of resource scarcity and supporting colony development under stressful conditions (DeGrandi-Hoffman et al., 2008). However, supplements sometimes do not meet all seasonal colony needs, possibly due to nutritional deficiencies (DeGrandi-Hoffman et al., 2016, DeGrandi-Hoffman et al., 2018), that could potentially be remedied with different mixtures and ratios of micronutrients (Bonoan et al., 2018).

We investigated dietary supplementation in Southern California during August, a typically warm and dry period with few floral resources. The local climate is Mediterranean and characterized by cool winters and warm, dry summers (Cowling et al., 1996). The flora is representative of coastal scrub, and important pollen resources for colonies during the dry season include native *Meleleuca viridiflora*, *Baccharis* spp., and several non-native eucalyptus species (Park and Nieh 2017).

Rather than studying complex diets that contain a mixture of various macronutrients (protein, carbohydrates, and fats) and micronutrients (sterols, minerals, and vitamins) that are found in pollen and nectar (Standifer 1980), we chose a diet containing sugar and amino acids (AA) only, which allowed us to focus on the nutritional effects of essential AA. Previously we found that free-flying foragers prefer

Abbreviations: AA, amino acid; EAA, essential amino acid; NAA, nonessential amino acid; P, protein; C, carbohydrate; -s, AAs, EAAs, NAAs, plural forms

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essential AA (EAA) over nonessential AA (NAA) (Hendriksma et al., 2014). Furthermore, foragers can meet their EAA needs by balancing an EAA deficient colony diet with a complementary diet (Hendriksma and Shafir, 2016). EAA can be deficient in bee-collected pollen: for example, arginine in dandelion (Herbert et al., 1970) and histidine in corn (Höcherl et al., 2012). Multi-EAA deficiencies may occur, as was noted for eucalyptus pollen (Manning, 2001), which could result in poor colony brood production (Loper and Cohen, 1987). How active nutrient supplementation by foragers may meet colony needs is a focus of current research and debate (Bonoan et al., 2018; Corby-Harris et al., 2018; Lihoreau et al., 2018).

AA are crucial for growth, development, and health of bees. Beside building blocks for protein synthesis, AA have multiple regulatory functions in cells. AA play important roles in regulating gene expression, cell signaling, antioxidative responses, and immunity (Wu 2010). De Groot (1953) identified the minimal requirements for 10 different EAA by raising bees in cages and measuring the threshold of growth impairment. Ten other AAs, also used for protein synthesis, can be synthesized by bees and are thus termed "non-essential." We therefore examined the effect of EAA supplementation on honey bee physiology, with NAA supplementation as a control treatment. Unlike other studies that performed tests with EAAs in equal concentrations (Archer et al., 2014, Paoli et al., 2014a,b, Simcock et al., 2014, Stabler et al., 2015), our test diet contained EAAs in the relative proportions required for honey bee growth (De Groot 1953). This arises from the rationale that the most limiting EAA is a bottleneck for growth (Filipiak et al., 2017). For example, honey bees need 4.5 times more leucine than tryptophan.

Whether EAA supplementation may help managed honey bees, particularly during seasons of high mortality (Rose et al., 2014; Steinhauer et al., 2014), deserves investigation. We hypothesized that colony nutrition can be supplemented with an AA diet and expected an increased growth of adult bees and brood following EAA treatment of colonies during the dry season, as compared to NAA treatment. We used a solid diet, in contrast to other studies that tested EAA nutrition effects with liquid diets (Archer et al., 2014, Hendriksma et al., 2014, Paoli et al., 2014a,b, Simcock et al., 2014, Stabler et al., 2015). Since colony protein nutrition is normally processed by nurses that consume beebread (i.e. a solid diet), we hypothesized that solid diet EAA supplementation would particularly benefit the nurses, more than foragers.

2. Material and methods

We studied diet effects on caged bees in the laboratory (n = 975 bees). At the same time, we performed a field study on how NAA/EAA supplementation affects colony brood development (N = 16 colonies) and on the different worker types, i.e., nurses and foragers (n = 800 bees). In a colony setting, a liquid diet is potentially stored away as honey. We thus used a solid diet to better reflect the uptake of protein, like from pollen (beebread), which is taken up almost exclusively by nurses, rather than by foragers (Crailsheim et al., 1992).

2.1. Honey bees

All experiments were conducted with queenright colonies (*A. m. ligustica*). Three colonies were used for our cage experiments and 16 colonies were used for colony level experiments. The colony AA supplementation experiments were performed at the Elliot Chaparral Reserve (32°53′37 N, 117°04″55 W). Colonies were standardized to contain five combs of brood in all stages of development, two combs filled with honey, and two empty combs at the outer sides (nine total combs). Based on counting combs with bees, and calculating 1215 bees per frame side as the average number of workers on a fully occupied side (Delaplane et al., 2013), our colonies were estimated to contain 19440–21870 workers.

Table 1
Amino acid profiles of pollen samples. Profile analyses of pollen with values of AA g/100 g pollen. Within curly brackets { }, the minimum dietary % of EAA per total AA is given (De Groot, 1953). Between square brackets [ ], we gave the % that each AA contributes to the total measured AA content. EAA nutrition provided by the pollen was not deficient because all [EAA] exceeded {EAA} values.

AA name	AA	Colony	Eucalyptus
Arginine {3.0}	Arg	1.11 [7.2]	1.45 [5.8]
Histidine {1.5}	His	0.40 [2.6]	0.68 [2.7]
Isoleucine {4.0}	Ile	0.66 [4.3]	1.08 [4.3]
Leucine {4.5}	Leu	1.12 [7.2]	1.73 [7.0]
Lysine {3.0}	Lys	1.00 [6.5]	0.83 [3.3]
Methionine {1.5}	Met	0.37 [2.4]	0.46 [1.8]
Phenylalanine {2.5}	Phe	0.73 [4.7]	0.99 [4.0]
Threonine {3.0}	Thr	0.54 [3.5]	1.11 [4.5]
Tryptophan {1.0}	Trp	0.26 [1.7]	0.40 [1.6]
Valine {4.0}	Val	0.84 [5.4]	1.43 [5.7]
Alanine	Ala	0.76 [4.9]	1.42 [5.7]
Asparagine	Asn	1.49 [9.6]	3.02 [12.1]
Cysteine	Cys	0.29 [1.9]	0.35 [1.4]
Glutamine	Gln	1.89 [12.2]	3.15 [12.7]
Glycine	Gly	0.67 [4.3]	1.17 [4.7]
Proline	Pro	2.02 [13.0]	3.25 [13.1]
Serine	Ser	0.75 [4.9]	1.42 [5.7]
Tyrosine	Tyr	0.59 [3.8]	0.94 [3.8]
Total AA in pollen		**15.5 [100]**	**24.9 [100]**

2.2. Diet preparation

Diets were designed at a different AA to carbohydrate ratios (Details in Table S1). The colony tested 1:17 AA:C ratio is lower than protein and carbohydrate intake based on consumption by incubated honey bees (e.g., Altaye et al., 2010). The difference relates to colonies having needs beyond maintenance of individual worker bees, such as communal breeding, building, transportation, and hibernation. Based upon the literature, we estimate that a normal-sized colony should consume 20 Kg pollen and 70 kg of honey in a year (Southwick and Pimentel 1981; Seeley et al., 1991; Brodschneider and Crailsheim 2010). Assuming a 20% AA content of bee pollen (Table 1), and a carbohydrate content of 80% for honey and 60% for bee pollen (Brodschneider and Crailsheim 2010, Lilek et al., 2015, Bertoncelj et al., 2018), we calculate that average annual colony intake is 4 kg AA versus 68 kg C: thus, a ratio of 1:17 AA:C.

Pure crystalline, alimentary grade AAs were purchased as L-form enantiomers (PureBulk, Roseburg, OR, USA). The EAA test diet was based on relative EAA proportions (w/w) required for bee growth (De Groot 1953). A mix of 10 g EAAs contained 1.07 g Arg + 0.54 g His + 1.43 g Ile + 1.61 g Leu + 1.07 g Lys + 0.54 g Met + 0.89 g Phe + 1.07 g Thr + 0.36 g Trp + 1.43 g Val. A mix of 10 g NAAs contained Ala + Asn + Asp + Cys + Glu + Gln + Gly + Pro + Ser + Tyr, all in equal proportions of 1.00 g each (w/w).

The diet preparation was as follows: we microwaved 179.1 g sugar candy (90/10 crème fondant, Mann Lake Ltd., Hackensack, MN, USA) until lukewarm (~40 °C). This was thoroughly mixed, using a handheld mixer, with 8.25 g of deionized water. Once a homogenous consistency was reached, 10 g AA mixture (EAA or NAA) was added, and mixed with the sugar paste for 1 min. Finally, 8.25 g powdered sugar was mixed in, resulting in a toffee-like consistency after cooling. Each final patty for colony application weighed 250 g, and was 12 × 20 × 1 cm in size. Each patty was contained in a 1000 ml plastic bag. For the cage experiments, feeders were 1.5 ml open Eppendorf tubes cut off at the tube tip to allow bee access to 3.28 g diet ± 0.02 SE. Diet preparations for cage and colony experiments were identical, but colony diets were prepared in 10 times larger batches.

In addition to 1:17 EAA or NAA to C, the cage experiments tested four additional control treatments to further test dose–response effects:

1:4 EAA to C, 1:50 EAA to C, 1:50 NAA to C, and a no-AA diet containing C only (a negative control). All treatment diet recipes are in Table S1 (Supplementary material).

2.3. Cage experiments

Brood combs without worker bees were incubated (Nor-Lake Scientific Incubator, model LRI201WWW/0) overnight in a nuc box at 34° C and 70% Relative Humidity (RH). The next day, 975 newly emerged bees were moved into transparent plastic cages (11 × 9 × 11 cm length × width × height, 25 bees per cage). Diet treatments were randomly assigned to cages, and each colony received all treatments. There were seven replicate cages for each diet treatment (Supplementary material; Table S2). Bees were supplied with two 5 ml syringes with water through the top of each cage and two feeding tubes with 6.6 g diet on the bottom of each cage. Diet tubes and water syringes were replaced at day 7.

All cages were incubated at 34° C and 70% RH for 14 days. We measured survival every day and any dead bees were recorded and removed. Diet consumption was determined by calculating the weight difference of feeding tubes before and after each week of feeding. We used survival and consumption data to calculate the mass of diet consumed per living bee per day. Several cages were excluded because of bee mortality, for example, due to water spills (Table S2).

2.4. Colony experiment

On August 1st 2017, each colony was given a 250 g patty: eight were given NAA and eight were given EAA, assigned randomly. Each patty bag was applied to colonies by placing it on top of brood frames and slicing open the bags with a knife to allow bee access. We provided the second patty on August 4th because we observed that > 90% of the initial patties had been consumed. With the approximate feeding rate of 3.8 g diet·h^{-1}, the 500 g treatment diets were fully consumed within a week. Averaged over 20 thousand bees, this is roughly 2 mg AA per bee (33 mg diet per bee).

On August 15th, 14 days after the start of diet exposure, 800 bees (50 per colony) were collected for weight measurements. Nurse bees were targeted by collecting 25 bees from a central brood comb containing open brood. Forager bees were collected by capturing 25 returning bees after closing off the hive entrance. Because pollen-carrying bees, identified by the pollen that they have in their corbiculae, were clearly foragers, we preferentially collected pollen-bearing bees (65%) over non-pollen-bearing bees (35%).

On August 23rd, capped brood cell numbers in all colonies were assessed. Each comb in the colony was checked, and sides containing capped brood were photographed. Using graphics software, we overlaid a grid of 8 squares over 191 comb photos. The percentage of capped brood cell coverage was estimated for each square and then multiplied by the cell count per square (419 cells per 115 cm^3). These results were combined to estimate the total number of capped brood cells per colony.

2.5. Bee body weight analyses

We studied how essential AA supplementation differentially affected the weight of worker body parts. Instead of measuring total bee weights (De Groot 1953), we measured body part weights (head, thorax, and abdomen) because different bee castes develop different parts of their bodies. Nurse bees need to develop hypopharyngeal (brood food) glands in their heads, but forager bees need strong thoracic flight muscles to collect pollen and nectar (Brodschneider and Crailsheim 2010).

Bees were collected on dry ice, and body parts were separated with iris micro scissors at the head, thorax (including legs and wings) and abdomen. The fresh body parts were immediately weighed in a Mettler Toledo scale (accurate to 0.0001 g) to obtain fresh weights (COLOSS BEEBOOK §2.2.4.; Human et al., 2013). Any corbicular pollen on forager bees was removed with tweezers prior to dissection, and kept for later analyses. The weight of the abdomen included the gastrointestinal tract. The dry weight of a single bee head was at the accuracy limit of the scale. We therefore pooled the body parts of five bees per treatment, measuring the average body part weight to improve accuracy. To obtain dry body part weights, we placed the open sample tubes at 60° C for seven days (Henderson 1992) in a ventilated incubator (Fisher Biotech). We dissected 1500 bees in total (Table S2; Supplementary material).

2.6. Pollen analyses

We collected 301 pollen pellets from the legs of sampled foragers during the field experiment. These pellets were sorted into the predominant color-morphs (187 beige, 88 brown, 24 orange, 2 grey-pink). Examination by light microscopy at 400X magnification (Leica stereoscope) revealed all brown pollen pellets (29%) to be *Eucalyptus* ssp., as identified by the typical triangular parasyncolpate shape.

To test for a potential EAA deficiency, we analyzed the nutritional quality of two pollen samples (field pollen and eucalyptus pollen). With regard to the observed ratio of color-morphs, we analyzed a homogenized blend of the polyfloral field collected bee pollen. The second pollen sample, monofloral eucalyptus pollen, was purposefully sorted out from a batch of regional pollen pellets (purchased from Sun Star Organics, Orange, CA). This pollen had the same appearance described above, and, with the help of Dr. Kale Sniderman (University of Melbourne, Victoria, Australia), we identified these pollens as belonging to the genus *Eucalyptus* (Sniderman et al., 2018).

AA-profiles of our pollen samples were obtained at the SPARC BioCentre (Toronto, ON). In preparation for Ultra-Performance Liquid Chromatography, the pollen pellet samples were crushed and homogenized. A 10 mg subsample was hydrolyzed (24 h at 110 °C) with 6 N hydrochloric acid (+1% phenol and norleucine as standard). Supernatant aliquots (10 µL) were dried, treated with a re-drying solution, vortex-mixed, vacuum dried (15 min) and derivatized (20 min). Diluted aliquots were then injected into the column at 48 °C, running a PICO-TAG gradient at 254 nm. The AA-profiles were based on quantifying single AAs, providing relative measures for 20 AAs (Table 1).

2.7. Statistical analyses

Statistical analysis was performed with JMP Pro 13.1.0 software. We conducted residuals analysis to ensure that the data conformed to parametric assumptions. The effect of diet supplementation on colonies was assessed with Analysis of Covariance (ANCOVA) on capped brood cell numbers per colony (response variable), with treatment as a factor (2 levels; EAA or NAA), and the total dry weights of nurse and forager bees as continuous variables (Fig. 1). We began with a full model but excluded interactions if they were not significant.

We used Analysis of Variance (ANOVA) to test the effect of diet treatments (10-level factor) on the dry weight data of body parts: head, thorax, and abdomen. A post hoc Tukey's Honestly Significant Difference (HSD) test was used for pairwise comparisons (Fig. 2).

Consumption by caged bees was analyzed with ANOVA with a Tukey HSD test (Fig. 3). We used Kaplan-Meier Survival analyses to determine the effect of the five AA treatments on survival of bees in cages. Wilcoxon post hoc tests were performed with the control, and we applied a correction for multiple comparison (Dunn–Šidák; $k = 5$, $\alpha = 0.010$).

3. Results

Strong differences were found between AA treatments, with respect to dry weight of bee heads (Fig. 2D; $F_{9,277} = 31.0$, $p < 0.001$), thoraces (Fig. 2E; $F_{9,277} = 38.1$, $p < 0.001$), and abdomens (Fig. 2F;

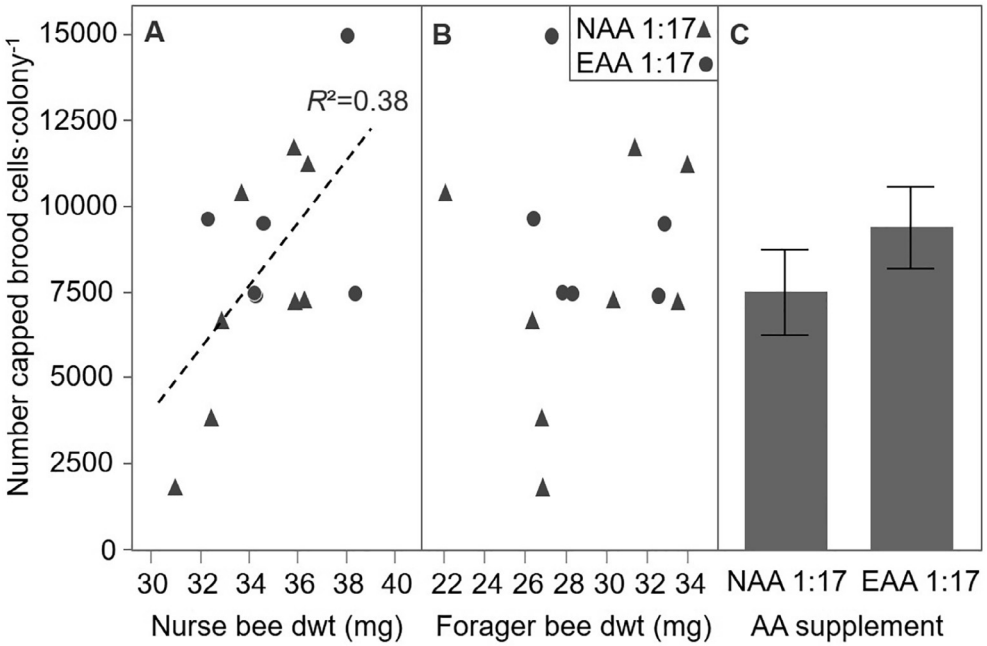

Fig. 1. Capped brood cell counts in colonies in relation to the weight of nurses, foragers, and essential amino acid (EAA) and non-essential amino acid (NAA) treatments. Colony treatment during the dry season in Southern California was a fondant diet with a 1:17 AA to sugar ratio, using either EAA or NAA. Mean capped brood cell numbers per colony are indicated with triangles (NAA) and dots (EAA). The mean nurse bee dry weights (dwt) correlated with colony brood production (A; p = 0.03), whereas the mean forager dry weights did not (B; p = 0.59). The number of capped brood cells in colonies did not differ between EAA and NAA treatment (C; bar plot, mean ± SE; p = 0.56).

$F_{9,277} = 68.0$, $p < 0.001$). In our colony experiments, we found a strong difference between foragers and nurses (Fig. 2 and Fig. 4). As compared to nurses, foragers had significantly lighter heads (16% dry weight, DW; 24% fresh weight, FW), but heavier thoraces (7% DW). Foragers had significantly lighter abdomens (48% DW; 65% FW), as compared with nurses.

Due to swarming or absconding, two EAA colonies were excluded from the capped brood cell analyses (Fig. 1). High nurse bee weights correlated with higher numbers of capped brood cells in colonies (Fig. 1, A; $R^2 = 0.38$, $F_{2,13} = 5.61$, $p = 0.03$). The amount of capped brood in colonies doubled (Fig. 1A; i.e. 5000 to 10,000 cells; +100%) when nurse bee weight increased by 5.3 mg (+17%). The relation between nurses and brood was the same for the EAA and NAA treatment (Interaction nurse weight × treatment; Fig. 1, A; $F_{3,12} = 1.42$, $p = 0.26$). Forager weights (Fig. 1, B; $F_{2,13} = 0.31$, $p = 0.59$) and AA treatment (Fig. 1, C; $F_{2,13} = 0.37$, $p = 0.56$) did not significantly affect brood production.

Within colonies, EAA supplementation was associated with slightly higher mean nurse bee head, thorax, and abdomen weights (+3%, +3%, +5%, respectively), but these trends were not significant (Fig. 2; Online graphical abstract). However, much larger effect sizes were observed in our caged bee experiments. As compared to NAA diet, the EAA diet significantly increased bee head, and thorax, and abdomen weights, by +35%, +15% and +19%, respectively (Fig. 2 and Fig. 4).

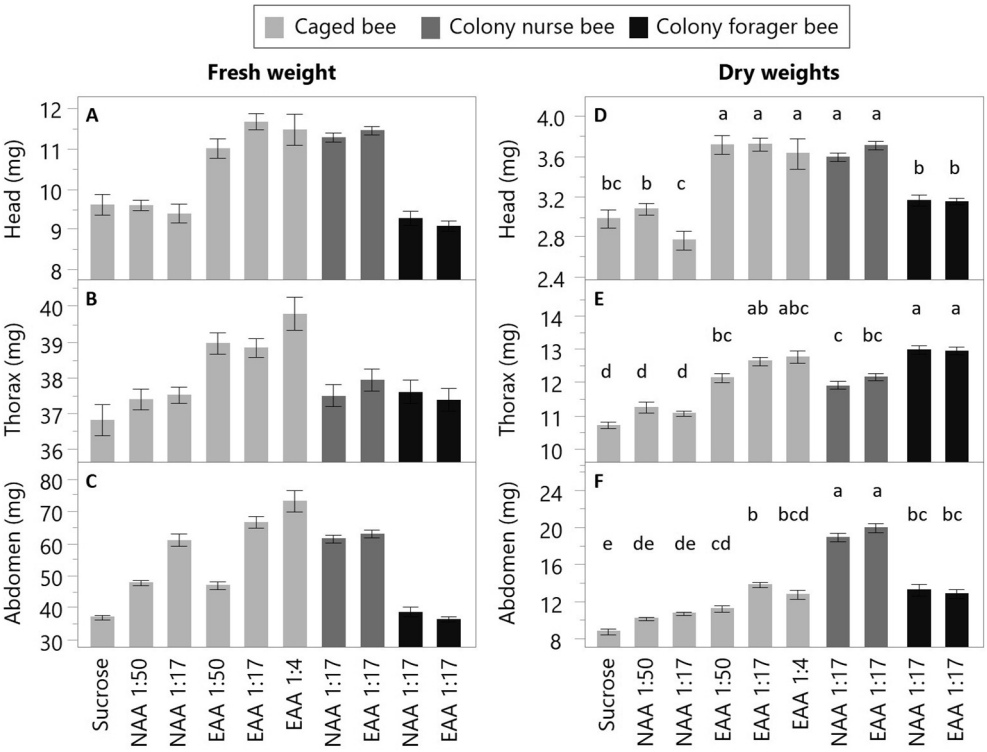

Fig. 2. Effects of EAA and NAA treatments on weights of honey bee body parts of bees from caged and colony experiments. Fresh weight (A, B and C) and dry weight (D, E and F) are shown for heads, thoraces, and abdomen. Different letters above the dry weight bars indicate significant differences (Tukey HSD tests). Diet treatments were different AA ratios to sugar, including a negative control (sucrose only) for the cage experiments.

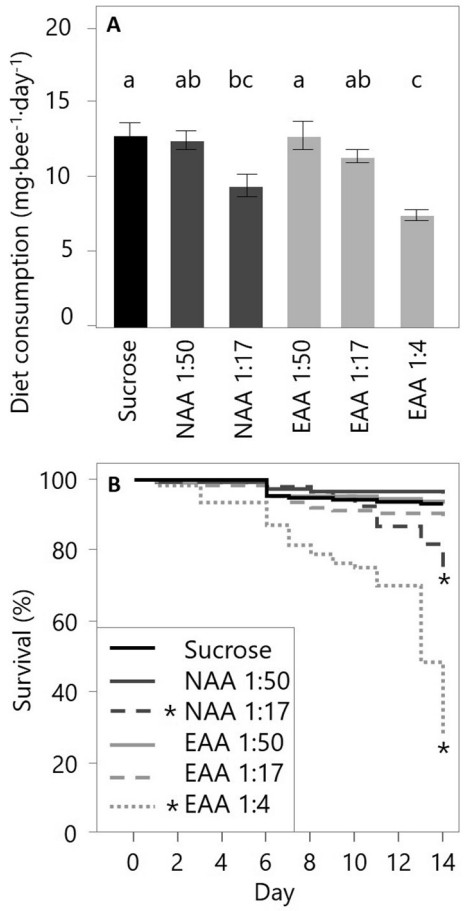

Fig. 3. Caged honey bee diet consumption and survival. The consumption of treatment diets (A) was calculated by dividing the total weekly diet consumed by the total number of bees alive. Different letters above the dry weight bars indicate significant differences (Tukey HSD tests). Daily bee mortality data were recorded to analyze treatment diet effects on honey bee survival (B). As compared to the sucrose control, 1:17 NAA and 1:4 EAA:C treatments showed a significantly lower survival (*).

NAA feeding to caged bees was similar to feeding no AA at all (Fig. 2D, E and F).

We observed several AA dose-response trends in the cage experiment (Fig. 2 and Fig. 3). As compared to the control, bee head weights plateaued for treatments of 1:50, 1:17 and 1:4 EAA (Fig. 2; D). In contrast, thorax and abdominal weights increased, according to increasing dietary AA concentrations (Fig. 2; E and F). AA treatment in cages significantly affected diet consumption by bees ($F_{5,26} = 7.79$, $p < 0.001$). Bees consumed less of the diet when the AA concentration was higher (Fig. 3, A). Honey bee survival in cages was significantly affected by the AA treatments (Wilcoxon $\chi 2 = 219.1$, $Df = 5$, $p < 0.001$). As compared to sucrose control treated bees (Fig. 3, B; 6.9% mortality), a 3-fold higher mortality was observed for 1:17 NAA to sucrose (21.4% mortality; $\chi 2 = 12.4$, $Df = 1$, $p < 0.001$) and 10-fold higher mortality for 1:4 EAA to sucrose (72.0% mortality; $\chi 2 = 95.4$, $Df = 1$, $p < 0.001$).

A summary figure illustrates the differences between bee body parts in colonies and cages, regarding our 1:17 AA to sucrose supplementation treatments (Fig. 4). When comparing caged bees to colony bees, we found that the head weights of caged bees on the EAA diet were similar to the head weights of colony nurses (Fig. 2D; letters "a"). Colony foragers were also similar to the caged bees on the 1:17 EAA diet with respect to their thorax (Fig. 2E; letters "a") and abdomen weights (Fig. 2F; letters "b"). The 1:17 NAA diet resulted in caged bees with stunted growth: they were significantly lighter than the colony bees (Fig. 2D, E, and F; letters c, d and e, respectively).

A key premise of testing EAA supplementation under field conditions was to counter potential poor pollen nutrition during summer dearth. In our field study, 29% of the collected pollen was from *Eucalyptus* ssp. (see Section 2.6). However, none of our analyzed pollen samples revealed EAA deficiency (Table 1).

4. Discussion

Given the progressive loss of sustainable pollinator habitats (Otto et al, 2016), there is a need to study how nutrient deficiency affects pollinators. Studies can illuminate how different pollen sources meet honey bee colony nutritional needs (Di Pasquale et al., 2016; Corby-Harris et al., 2018), particularly with respect to EAA (McCaughey, 1980; Rayner et al, 1985) and essential fatty acid levels (Avni et al, 2014, Zarchin et al., 2017, Arien et al., 2018). We found that poor bee nutrition (EAA deficiency; no-AA or NAA only) can be ameliorated with EAA diet in cages (Fig. 4). Caged bees fed 1:17 EAA showed a significant growth increase, and developed head weights similar to colony nurse bees, and thorax weights similar to colony foragers. Overall, our colony study suggested that nutrition may drive colony growth via nurse bees. There was a significant correlation ($R^2 = 0.38$) such that colonies with 17% heavier nurses also reared 100% more brood (Fig. 1A). Thus, we speculate that even modest increases in individual

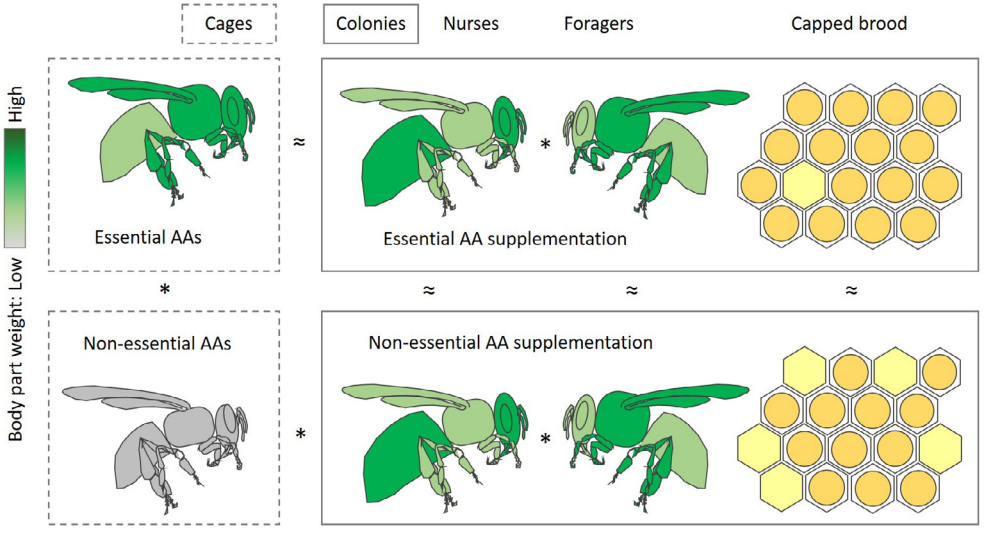

Fig. 4. Summary of EAA and NAA nutrition effects of body part dry weights of bees from caged and colony experiments. Results are illustrated treatment effects by 1:17 AA to sucrose diet in cages (dashed line boxes) and in colonies (solid line boxes). Statistical differences are illustrated with four color levels, from low to high: grey, light green, green, and dark green. Statistical differences are also marked by asterisks (*) while similarity is shown with a double tilde (≈). As illustrated by comb drawings, capped brood cell (orange circles) counts were on average $^{EAA}9439$ and $^{NAA}7554$, though this 20% deviation by AA treatment was not statistically significant ($p > 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bee nutritional status may result in heavier bees and potentially improve colony productivity.

Despite significant differences between EAA and NAA supplementation in cages, we found no effect in field colonies. Specifically, we found no significant treatment difference in colony brood cell numbers (Fig. 1C; ^{EAA}9439 versus ^{NAA}7554) or in nurse and forager body part weights (Fig. 2). We suggest this occurred because field colonies were not significantly depleted of EAA since foragers were bringing in pollen from the surrounding environment. Colony foragers may compensate for nutritional deficiencies by shifting foraging preferences (Hendriksma and Shafir, 2016). Also, nitrogen content and the dry weight of bees increases during the first 10 days of a bee's life, but stabilizes thereafter (De Groot 1953). Hence, for the relatively young caged bees, as compared to older colony bees, the difference in potential growth may have led to the more pronounced effect size in cages as compared to AA treatment in colonies.

The original premise of our EAA supplementation was to counter what we expected to be poor pollen nutrition based on available field forage. Eucalyptus pollen had previously been described to be EAA and fatty acid (FA) deficient (Bell et al., 1983; Manning 2001; Somerville and Nicol, 2006, Arien et al., 2015), providing the rationale for testing EAA supplementation (Szymaœ and Maliszewska, 1999; Rogala and Szymás, 2004). We assumed, based upon the literature, that eucalyptus pollen would be an important pollen source for colonies during the dry season, which we corroborated by 1 in 3 pollen pellets being from eucalyptus trees. However, contrary to our expectations, we found no AA deficiency in the monofloral eucalyptus pollen, or in the pollen mixture collected by colony bees (Table 1). The eucalyptus pollen protein content of 24.9% AA (w/w) pollen was notably high in comparison with the 15.5% AA (w/w) of field collected pollen (Table 1). These results suggest that eucalyptus pollen may be a good source of pollen protein for honey bee colonies during dry season, a period of relative pollen dearth.

A striking physiologically-based worker caste distinction is illustrated by nurse and forager bees having significantly different weights for all three measured body parts (Fig. 4). Nurses had significantly heavier heads (Fig. 2, A and D), likely because of hypopharyngeal brood food glands (Hrassnigg and Crailsheim 1998; Omar et al., 2017). As compared to the nurses, foragers had significantly heavier thoraces (Fig. 2, B and E), likely due to an increase in flight muscle mass. Prior studies have shown that thoracic development and subsequent foraging performance are reduced when worker bees are nutritionally limited (Scofield and Mattila 2015). Nurse abdomens were significantly heavier than those of foragers. This could arise from differences in gut contents (nurses with recently consumed pollen and retained fecal matter) as well as from differences in fat and protein stores (Toth and Robinson 2005). Pollen consumption by foragers is much lower (Crailsheim et al., 1992), and frequent defecation may reduce abdominal weight, and thus the caloric cost for flight (Moffatt 2000).

Although we found no significant difference between EAA and NAA supplementation in colonies (Fig. 1), it is still possible that supplementing AA in colonies contributed to overall colony growth. However, the effect size of colony growth due to either the EAA or NAA treatment could not be directly assessed, in lieu of a no-AA or no-supplementation control. Yet under caged conditions, each bee consumed approximately 10 mg EAA (Fig. 3; 12 mg diet per day for 2 weeks) and increased 8.6 mg (DW) in weight, as compared to the NAA and no-AA diets. This nutrient to body mass conversion rate (i.e., mg AA into mg bee), if scaled up to our colony conditions (500 g diet * 1:17 ratio = 29.4 g AA), suggests a colony growth potential of 25.3 g (DW) bee mass growth within colonies. This is hypothetically equivalent to 1278 new born bees (DW * 19.8 mg bee^{-1}; Hrassnigg and Crailsheim, 2005). These calculations suggest that colony growth could be stimulated by AA supplementation.

The cage experiment illustrates the importance of essential amino acids, and AA supplementation, despite a lack of treatment effects in colonies (Fig. 4). Our laboratory trials were performed under worst case conditions of absolute AA deficiency. As compared to feeding with no AA, the caged bees that were fed NAA did not grow. As compared to no AA or NAA feeding, we found approximately 1.5-fold higher weights for head, thorax, and abdomen when caged bees were fed EAA. These results are consistent with young bees having a nitrogen content of 1.74 mg/bee upon emergence, which increased to 2.65 mg/bee after three weeks (Haydak, 1934), a 1.5-fold increase weight.

In the absence of brood, caged bees may invest in personal growth such as in fat, glandular and muscle tissue (Eyer et al., 2017). In addition, queenless bee groups establish social hierarchies, with dominant bees showing ovariole and mandibular gland development that reinforce their domination hormonally and pheromonally, as part of reproductive competition (Altaye et al., 2010, Crewe and Moritz 1989, Pirk et al., 2010). Simon et al. (2001) found that young workers of the Cape honey bee (*A. mellifera capensis*) rapidly increased mandibular gland secretions after becoming queenless. Within our cage experiments, the effects of such reproductive competition may explain some variation in body part weight. However, it does not account for the large observed differences between the treatment groups because queenlessness occurred in all treatment groups in this experiment. The weight effects observed our caged bee experiment were thus evidently driven by treatment effects, not by queenlessness. Unusually, caged 14-day old EAA treated bees had head weights and thorax weights that were respectively similar to those of colony nurses and foragers (Fig. 4). It is not clear why this occurred, but perhaps the absence of larvae to feed resulted in increased hypopharyngeal gland size, while thorax weight increased, as appropriate for bees approaching foraging age.

Caged bees consuming AA in solid sucrose diets showed a notable dose response trend: bees ate more of the sucrose diet and the diet with the 1:50 AA:C ratios, as compared to the 1:17 diets, but ate the 1:4 diet the least (Fig. 3A). Diet consumption according to an AA intake target may explain this trend. At a high AA concentration diet of 1:4 AA:C a small amount may suffice for bee AA needs, while at a lower 1:17 concentration, more diet may need to be consumed. Although our diet treatments altered the physiology of caged bees (Fig. 4), except for the 1:4 AA:C diet, there were no significant effects of treatment on consumption rates (Fig. 3A). This suggests that the nutritional status of bees did not strongly affect their diet consumption under the conditions tested. Instead, bee preferences seemed to explain diet consumption. Bees either preferred the low AA concentration or perhaps found the high AA concentration unappetitive or noxious.

Approximately 30% of calories from protein are spent in digesting it (Ganong 1969). Bees should therefore consume about 1.3 times more protein than their actual requirement for AA. For example, honey bee colonies deprived of pollen and honey stores under semi-field conditions collected protein to carbohydrate (P:C) at an average ratio of 1:14 (Hendriksma et al., 2019). Feeding three sources of protein in a paste diet to young caged honey bees resulted in similar intakes of 1:11 to 1:14 P:C (Altaye et al., 2010). With respect to the 1.3 factor, this translates into a range of 1:14 to 1:18 EEA:C. EAA fed in solution to young caged bees showed an intake of 1:50 EAA:C (Paoli et al., 2014a), which may indicate that bees are more willing to consume EAA in solids as compared to in solutes. We note that at 1:50 EAA, the growth of caged bees was suboptimal: their thoracic weight was significantly lower as compared to colony foragers (Fig. 2; E). We think that the 1:17 AA:C ratio tested on cage and colony bees in the current study is a realistic ratio because the 1:50 ratio is too low (resulting in suboptimal growth, Fig. 2), while the 1:4 ratio is too high (resulting in elevated mortality, Fig. 3B).

In our cage experiments the overall mortality was fairly low, except for bees fed a diet of 1:4 EAA:C and 1:17 NAA:C (Fig. 3). Potential bias due to bee mortality should therefore be low for all other treatments (Control, 1:50 NAA, 1:50 EAA and 1:17 EAA diets in cages, and the 1:17 NAA and 1:17 EAA diets in colonies). Our survival data is in line with a reported 100% bee mortality at 1:5 EAA:C and 33% mortality at 1:50

EAA:C (Paoli et al., 2014a,b). Mortality caused by high AA consumption is well known (Huang et al., 2011, Dussutour and Simpson 2012, Arganda et al., 2017). In response, bees may respond by reducing their intake of AA (Fig. 3A), possibly enabled by gustatory detection (De Brito Sanchez 2011, Hendriksma et al., 2014). When considering AA supplementation to support colony growth, the digestive benefit of feeding AA over protein needs to be weighed against the reluctance of bees to feed on AA and mortality effects at higher AA concentrations.

Our study also highlights the importance of testing honey bee nutrition with field and laboratory studies. Testing diet treatments on caged bees as compared with whole colonies may yield different insights (Paoli et al., 2014a,b, Lihoreau et al., 2015). Side-by-side studies of nutrient deficiency in cages and colonies can improve our understanding of how colonies respond to poor nutrition, how this impacts different colony members, and how dietary supplementation helps bees faced with combined stressors such as poor nutrition, pesticide exposure (Tosi et al., 2017) and pathogen infections (Dolezal and Toth, 2018).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2019.103906.

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Supplementary Information

Honey bee AA supplementation (Hendriksma et al. 2019)

Table S1: Diet recipes

Treatment[1]	Fondant[2]	Water	Amino Acids	Powdered sugar
Negative control	92.0 g	4.0 g	no AA	4.0 g
1:4 EAA to C	41.9 g	2.26 g	10 g EAA mix[3]	2.26 g
1:17 EAA to C	179.1 g	8.25 g	10 g EAA mix[3]	8.25 g
1:50 EAA to C	529.5 g	23.46 g	10 g EAA mix[3]	23.46 g
1:17 NAA to C	179.1 g	8.25 g	10 g NAA mix[4]	8.25 g
1:50 NAA to C	529.5 g	23.46 g	10 g NAA mix[4]	23.46 g

[1] The 1:17 AA to Carbohydrate ratio, chosen for colony supplementation and cage tests, was based on data of annual honey bee colony consumption of 70 kg honey and 20 kg pollen.

[2] The "90/10 crème fondant" contained the ingredients Sugar (90%) and High Fructose Corn Syrup (10%) and was purchased from Mann Lake Ltd, 5001 1st St S. Hackensack, MN 56452.

[3] The mix of 10 g EAAs contained 1.07 g Arg + 0.54 g His + 1.43 g Ile + 1.61 g Leu + 1.07 g Lys + 0.54 g Met + 0.89 g Phe + 1.07 g Thr + 0.36 g Trp + 1.43 g Val. These relative EAA proportions (w/w) follow the minimal requirements for honey bee growth, as reported by De Groot (1953). The pH of the EAA diets was slightly alkaline (8.08, 8.28 and 8.03, for 1:50, 1:17 and 1:4 EAA to C, respectively, for which we applied no correction to avoid the potential confounding factor of adding a pH correcting agent.

[4] A mix of 10 g NAAs contained Ala + Asn + Asp + Cys + Glu + Gln + Gly + Pro + Ser + Tyr, all in equal proportions w/w). The pH of the NAA diets was acidic (i.e., 3.36 for 1:50 NAA to C).

Supplementary Information

Honey bee AA supplementation (Hendriksma et al. 2019)

Table S2: Detailed data from experiment: sample sizes, survival, consumption, and mean weight per body part, both as fresh-weights and dry-weights (fw = fresh weight, dw = dry weight, n.d. = no data.)

EAA and NAA = Essential and Nonessential Amino Acids, respectively). EAA and NAA diet ratios are AA to C (C = Carbohydrate).

We dissected 1500 bees. Once pooled (5 bees per sample), per body part, this yielded 300 samples with heads, 300 with thoraces, and 300 abdomens. We then calculated average body part masses per bee.

Four cages were excluded from analyses (cages a13, b12, b13 and c13). The applied pollen based diet (1:17 AA content to sucrose) did not solidify and leaked from feeding tubes, sticking to bees. This leakage impaired the measurement of diet consumption and bee weights, and was cause of bee mortality.

Bees in cages / colonies	[7] Treatments	[19] Colonies	[39] Cages	[1775] Bees	Dead/Censored/Alive	Consumption (mg/bee/day)	[1474] Bees dissected	Heads fw (mg/bee)	Thorax fw (mg/bee)	Abdomen fw (mg/bee)	Heads dw (mg/bee)	Thorax dw (mg/bee)	Abdomen dw (mg/bee)	[900] H+T+A samples
Cage	Sucrose	A	a1	25	0/0/25	12.6	25	8.9	36.7	34.5	2.7	10.3	7.7	5+5+5
Cage	Sucrose	A	a2	25	5/0/20	11.2	20	8.1	34.4	32.4	2.6	10.2	7.1	4+4+4
Cage	Sucrose	B	b1	25	2/0/23	13.3	20	9.6	36.1	38.8	3.1	11.1	9.3	4+4+4
Cage	Sucrose	B	b2	25	1/0/24	n.d.	20	8.9	34.4	37.8	2.8	10.6	9.2	4+4+4
Cage	Sucrose	C	c1	25	3/1/21	13.5	20	11.1	38.7	40.0	3.1	10.9	9.2	4+4+4
Cage	Sucrose	C	c2	25	1/0/24	16.1	24	11.0	39.9	38.9	3.7	11.2	9.9	5+5+5
Cage	Sucrose	C	c3	25	0/0/25	10.6	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0+0+0
Cage	NAA 1/50	A	a3	25	1/0/24	10.4	25	10.2	36.6	43.8	3.3	10.6	9.7	5+5+5
Cage	NAA 1/50	A	a4	25	1/0/24	12.5	20	8.5	36.5	49.1	2.8	11.0	10.0	4+4+4
Cage	NAA 1/50	B	b3	25	3/0/22	12.9	20	9.0	36.0	46.8	3.0	11.0	10.2	4+4+4
Cage	NAA 1/50	B	b4	25	0/0/25	11.2	25	9.3	39.3	47.0	2.9	11.5	10.0	5+5+5
Cage	NAA 1/50	C	c4	25	1/0/24	14.2	24	9.5	37.7	51.0	3.0	11.6	10.4	5+5+5
Cage	NAA 1/50	C	c5	25	0/0/25	14.1	25	9.9	37.1	50.0	3.2	11.6	10.4	5+5+5
Cage	NAA 1/17	A	a4	25	11/0/14	n.d.	14	9.0	36.0	62.6	2.4	10.4	9.4	3+3+3
Cage	NAA 1/17	A	a6	25	2/6/17	9.1	17	9.5	38.1	63.2	2.6	10.7	10.6	4+4+4
Cage	NAA 1/17	B	b5	25	1/0/24	8.5	24	11.2	37.9	55.4	3.5	11.5	11.0	5+5+5
Cage	NAA 1/17	B	b6	25	8/0/17	7.7	15	8.8	38.3	61.6	2.6	11.2	10.4	3+3+3
Cage	NAA 1/17	C	c6	25	2/3/20	10.1	20	9.5	37.9	70.3	2.7	11.0	11.5	4+4+4
Cage	NAA 1/17	C	c7	25	11/0/14	12.1	14	8.3	37.6	69.0	2.4	11.5	11.8	3+3+3
Cage	EAA 1/50	A	a7	25	1/0/24	12.1	20	10.1	38.7	45.5	3.5	12.7	12.1	4+4+4
Cage	EAA 1/50	A	a8	25	5/0/20	11.7	20	10.1	38.8	43.7	3.3	11.8	10.4	4+4+4
Cage	EAA 1/50	B	b7	25	0/8/17	13.0	20	12.3	37.8	46.9	4.2	11.6	11.0	4+4+4
Cage	EAA 1/50	B	b8	25	3/0/22	9.9	20	10.9	39.2	41.8	3.5	11.8	9.6	4+4+4
Cage	EAA 1/50	C	c8	25	0/1/24	13.6	10	11.7	38.7	47.4	4.1	11.9	12.0	2+2+2
Cage	EAA 1/50	C	c9	25	1/0/24	16.8	20	11.4	40.6	56.6	3.9	12.9	12.8	4+4+4
Cage	EAA 1/17	A	a9	25	2/0/23	10.9	23	12.0	38.7	60.8	4.0	12.3	13.9	5+5+5
Cage	EAA 1/17	A	a10	25	4/0/21	10.1	20	12.1	38.5	60.0	3.7	12.1	13.8	4+4+4
Cage	EAA 1/17	B	b9	25	3/0/22	11.4	20	12.2	38.5	68.4	3.7	12.6	13.9	4+4+4
Cage	EAA 1/17	C	c10	25	4/0/21	12.2	20	10.2	37.8	76.8	3.5	13.2	14.5	4+4+4
Cage	EAA 1/17	C	c11	25	0/0/25	12.7	25	11.9	40.4	67.9	3.7	13.0	13.1	5+5+5
Cage	EAA 1/4	A	a11	25	7/5/13	7.6	13	12.2	38.9	71.8	3.9	12.4	12.8	3+3+3
Cage	EAA 1/4	A	a12	25	16/6/3	6.5	3	11.7	40.8	60.3	3.4	13.3	11.3	1+1+1
Cage	EAA 1/4	B	b10	25	14/6/5	7.8	5	10.5	40.4	82.4	3.2	13.2	13.6	2+2+2
Cage	EAA 1/4	B	b11	25	19/5/1	8.2	1	11.3	40.9	71.6	3.8	12.6	12.3	1+1+1
Cage	EAA 1/4	C	c12	25	8/17/0	n.d.	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0+0+0
Cage	Pollen 1/17	A	a13	25	3/2/20	30.6	20	11.0	38.5	79.0	3.9	13.8	26.0	4+4+4
Cage	Pollen 1/17	B	b12	25	8/0/17	19.7	17	11.1	40.5	81.4	4.0	15.1	25.1	4+4+4
Cage	Pollen 1/17	B	b13	25	0/0/25	18.4	25	10.5	39.7	71.6	3.6	13.8	23.2	5+5+5
Cage	Pollen 1/17	C	c13	25	16/6/3	26.4	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0+0+0
Colony	EAA 1/17	D	Foragers	25			25	8.3	35.5	31.8	2.9	12.7	12.0	5+5+5
Colony	EAA 1/17	E	Foragers	25			25	8.5	35.9	33.7	3.1	12.3	10.7	5+5+5
Colony	EAA 1/17	F	Foragers	25			25	9.2	39.0	36.6	3.2	13.8	16.5	5+5+5
Colony	EAA 1/17	G	Foragers	25			25	9.3	39.4	38.6	3.2	13.5	12.0	5+5+5
Colony	EAA 1/17	H	Foragers	25			25	8.3	36.0	34.8	3.1	12.5	11.7	5+5+5
Colony	EAA 1/17	I	Foragers	25			25	10.5	38.8	40.9	3.4	12.8	12.0	5+5+5
Colony	EAA 1/17	J	Foragers	25			25	8.6	36.5	33.8	3.0	12.5	11.2	5+5+5
Colony	EAA 1/17	K	Foragers	25			25	9.6	38.1	40.9	3.3	13.1	16.8	5+5+5
Colony	EAA 1/17	D	Nurses	25			25	11.5	36.2	69.1	3.7	11.7	22.7	5+5+5
Colony	EAA 1/17	E	Nurses	25			25	11.0	36.3	62.1	3.8	12.5	20.3	5+5+5
Colony	EAA 1/17	F	Nurses	25			25	11.0	39.6	59.8	3.6	12.4	18.6	5+5+5
Colony	EAA 1/17	G	Nurses	25			25	11.4	38.3	74.1	3.6	12.1	22.7	5+5+5
Colony	EAA 1/17	H	Nurses	25			25	11.4	39.0	59.0	3.8	12.6	20.9	5+5+5
Colony	EAA 1/17	I	Nurses	25			25	12.3	39.4	62.1	3.8	12.4	18.0	5+5+5
Colony	EAA 1/17	J	Nurses	25			25	11.1	36.0	60.2	3.5	11.3	17.6	5+5+5
Colony	EAA 1/17	K	Nurses	25			25	12.2	38.9	57.7	3.9	12.4	18.0	5+5+5
Colony	NAA 1/17	L	Foragers	25			25	9.1	38.3	41.2	3.3	14.1	16.8	5+5+5
Colony	NAA 1/17	M	Foragers	25			25	8.8	37.3	32.1	3.1	13.0	10.6	5+5+5
Colony	NAA 1/17	N	Foragers	25			25	10.5	39.9	51.1	3.4	13.2	18.1	5+5+5
Colony	NAA 1/17	O	Foragers	25			25	9.4	39.2	42.2	3.0	13.2	14.6	5+5+5
Colony	NAA 1/17	P	Foragers	25			25	9.1	38.8	36.6	3.1	13.3	10.8	5+5+5
Colony	NAA 1/17	Q	Foragers	25			25	11.0	38.3	50.9	3.8	13.0	15.1	5+5+5
Colony	NAA 1/17	R	Foragers	25			25	8.7	35.6	33.8	3.0	12.4	11.8	5+5+5
Colony	NAA 1/17	S	Foragers	25			25	7.7	33.6	24.1	2.7	11.6	7.9	5+5+5
Colony	NAA 1/17	L	Nurses	25			25	11.9	38.6	75.9	3.8	12.4	19.6	5+5+5
Colony	NAA 1/17	M	Nurses	25			25	11.1	37.8	54.6	3.6	12.5	16.8	5+5+5
Colony	NAA 1/17	N	Nurses	25			25	12.1	40.8	62.4	3.8	12.9	19.7	5+5+5
Colony	NAA 1/17	O	Nurses	25			25	11.1	36.4	65.5	3.6	11.6	21.2	5+5+5
Colony	NAA 1/17	P	Nurses	25			25	10.6	38.7	56.2	3.3	11.8	17.4	5+5+5
Colony	NAA 1/17	Q	Nurses	25			25	11.8	37.6	66.6	3.8	12.1	20.0	5+5+5
Colony	NAA 1/17	R	Nurses	25			25	11.4	35.1	55.1	3.5	10.8	16.6	5+5+5
Colony	NAA 1/17	S	Nurses	25			25	10.4	35.1	56.5	3.3	11.2	19.2	5+5+5