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RESEARCH ARTICLE

Prisoners receive food fit for a queen: honeybees feed small hive beetles protein-rich glandular secretions through trophallaxis

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ABSTRACT

The honeybee nest parasite *Aethina tumida* (small hive beetle) uses behavioural mimicry to induce trophallactic feeding from its honeybee hosts. Small hive beetles are able to induce honeybee workers to share the carbohydrate-rich contents of their crops, but it is not clear whether the beetles are able to induce to workers to feed them the protein-rich hypopharyngeal glandular secretions fed to the queen, larvae and other nest mates. Protein is a limiting macronutrient in an insect's diet, essential for survival, growth and fecundity. Honeybees obtain protein from pollen, which is consumed and digested by nurse bees. They then distribute the protein to the rest of the colony in the form of hypopharyngeal gland secretions. Using ^{14}C-phenylalanine as a qualitative marker for protein transfer, we show that small hive beetles successfully induce worker bees to feed them the protein-rich secretions of their hypopharyngeal glands during trophallaxis, and that females are more successful than males in inducing the transfer of these protein-rich secretions. Furthermore, behavioural observations demonstrated that female beetles do not preferentially interact with a specific age cohort of bees when soliciting food, but males tend to be more discriminant and avoid the more aggressive and active older bees.

KEY WORDS: *Aethina tumida*, 14C-phenylalanine, Hypopharyngeal gland, *Apis mellifera*, parasite

INTRODUCTION

The small hive beetle (SHB), *Aethina tumida*, is a scavenger and nest parasite in colonies of western honeybee (*Apis mellifera*). These beetles reproduce in bee nests while feeding on bee brood, honey and pollen stores, but seldom inflict serious damage on honeybee colonies in its native range in sub-Saharan Africa (Ellis and Hepburn, 2006; Hepburn and Radloff, 1998; Lundi, 1940; Neumann and Elzen, 2004). In contrast, outside of its native range, the SHB is proving to be an economically important and deleterious parasite of social bee colonies, including honeybees, bumblebees and stingless bees (Greco et al., 2010; Hoffmann et al., 2008; Neumann and Ellis, 2008; Spiewok and Neumann, 2006).

The observed resistance to SHB infestations by honeybee subspecies native to Africa is most likely due to quantitative differences in a series of behaviours including aggression, absconding, removal of beetle eggs and larvae, and social encapsulation (walling) of invading beetles in cracks and crevices in the hive using propolis (Elzen et al., 2001) that developed in the course of co-evolution. Social encapsulation is a highly sophisticated defensive behaviour displayed by honeybees that restricts the SHB's access to the honey, pollen and protein-rich bee brood in the combs, ultimately limiting and postponing successful beetle reproduction within the hive (Ellis, 2005; Ellis et al., 2003a, 2003c; Neumann et al., 2001b). Both African and European honeybee subspecies encapsulate SHBs, but the social encapsulation efforts of the European honeybees do not successfully contain SHBs below detrimental levels (Ellis et al., 2003a,b,c). SHBs, in turn, survive in the hive by escaping, hiding, dropping (to the ground), assuming a turtle-defence posture and laying eggs in small gaps or crevices that bees cannot access (Neumann et al., 2001b). SHBs even survive the imprisonment (with no access to food) for 2 months or longer as they are able to induce trophallactic feeding by their honeybee guards through behavioural mimicry (Ellis et al., 2002; Neumann et al., 2001b).

Trophallaxis (the exchange of liquid food between nest mates) is a common interaction in social insects and serves as a source of nutrition and communication cues (Crailsheim, 1998). Trophallactic interactions between a parasite and its host in social insect colonies are not unique to honeybees and are known to occur in ant (Hölldobler and Wilson, 1990) and termite (Howard et al., 1980) colonies as well. The parasites use chemical and acoustic mimicry to disguise themselves in the host colonies as nest mates and use tactile stimuli to induce feeding (Barbero et al., 2009; D'Ettorre et al., 2002; Howard et al., 1980; Moritz et al., 1991; Schmid-Hempel, 1998). Interestingly, current empirical evidence suggests that SHBs do not rely on chemical mimicry to avoid aggression by host worker bees (Neumann et al., 2015; Pirk and Neumann, 2013). Behavioural studies (using nucleus hives with SHBs separated from the bees with metal gauze that prevented mingling but allowed antennal and mouthpart contact, or bees and SHBs caged together) found that when soliciting food, a SHB mimics bee–bee trophallaxis by approaching a worker bee, extending its heads and making antennal contact with the bee (Ellis et al., 2002; Neumann et al., 2015; Pirk and Neumann, 2013). The SHB uses its mouthparts and forelegs to touch the bee's head, mandibles and antennae anteriorly and posteriorly (Neumann et al., 2015), displaying a 'begging behaviour'. This behaviour frequently provokes an aggressive reaction from the worker bee, which tries to grab the SHB with its mandibles (Neumann et al., 2015). Begging events are typically separated by breaks, and during these breaks the worker bee vigorously attacks the SHB (Elzen et al., 2001; Neumann et al., 2015). Unlike other chemically disguised honeybee nest parasites such as the death head's hawkmoth (Moritz et al., 1991) and bee louse (Martin and Bayfield, 2014), SHBs risk injury when interacting with honeybee workers, as they are easily detected and subsequently attacked (Elzen et al., 2001; Neumann et al., 2015). The fact that SHBs are readily recognised as non-nest mates and attacked by host worker bees does not entirely exclude the possibility that some form of olfactory mimicking may be present during SHB trophallactic solicitation.

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Trophallactic solicitation or begging in SHBs is an innate behaviour that can be influenced by the beetle's sex and experience (Neumann et al., 2015). In contrast, the success rate, feeding and begging durations are not significantly affected by either sex or experience (Neumann et al., 2015), as the likelihood of a honeybee worker to feed, as well as the amount of food transferred, depends largely on the donor bee's nutritional state (Crailsheim, 1998; Free, 1956). Pirk and Neumann (2013) demonstrated that SHBs display differential behaviour toward young (<48 h old), less aggressive and older (>20 days), more aggressive worker bees, indicating that SHBs are able to assess the defensiveness of the host and adjust their behaviour accordingly. In addition, the possibility exists that SHBs are able assess the bee's nutritional state and only beg from bees with a higher nutritional state, as the overall solicitation success rate is rather high (∼40%) (Ellis et al., 2002).

During trophallaxis, honeybee workers can transfer either a drop of honey regurgitated from their crops, or jelly, a protein-rich glandular secretion. The primary source of protein in a honeybee colony is pollen and its main consumers are nurse bees, the young workers that digest the pollen and produce the protein-rich jelly in their hypopharyngeal glands. Nurse bees feed this jelly to the larvae and queen, but also to nest mates, including the drones, through trophallactic interactions (Crailsheim et al., 1992; Wright et al., 2018). Protein is critical for the survival and reproduction of honeybees; likewise, it is also a fundamental part of the SHB's diet. This leads to the subsequent question: do bees share the protein-rich glandular secretions with SHBs during trophallactic interactions? Previously, Ellis et al. (2002) demonstrated and confirmed that bees share their crop contents with SHBs during these interactions, but it is not known whether bees share the valuable protein-rich jelly with the SHBs as well. Furthermore, in comparison with the other age cohorts of bees in the hive, nurse bees have higher nutritional status as distributors of the protein-rich jelly. Considering that the success of trophallaxis is dependent on the donor bee's nutritional status (Crailsheim, 1998; Free, 1956), do SHBs then preferentially solicit food from nurse bees?

In this study, we attempt to answer these questions by investigating whether male and female SHBs preferentially target a specific age cohort of bees for food when given a choice between newly emerged bees, nurse bees and foragers. We predicted that female SHBs would more readily risk aggressions to receive protein-rich jelly from nurse bees due to the females' higher protein requirements owing to egg production and often larger body size (Lundi, 1940; Neumann et al., 2015), whereas males would have less of a preference when targeting honeybees for food. In addition, we measured whether proteinaceous secretions from the hypopharyngeal glands are transferred to SHBs during trophallactic interactions using ^{14}C-labelled phenylalanine as a qualitative marker for protein transfer (Crailsheim, 1998) to SHBs.

MATERIALS AND METHODS

Small hive beetles

Adult SHBs, *Aethina tumida* Murray 1867, were randomly collected from colonies of *Apis mellifera scutellata* Lepeletier 1836 located on the experimental farm of the University of Pretoria (25°45′13.6″S, 28°15′29.0″E, Pretoria, South Africa), sexed and used to start a laboratory colony. The SHBs were kept in plastic containers (40×30×30 cm) at 28±1°C and supplied with wax comb, honey, pollen, nectar and additional pollen patties (pollen substitute:honey in a ratio of 3:1), according to standard rearing protocols (Neumann et al., 2001a). Wandering larvae (after ∼18 days) were moved to pupation containers containing autoclaved, moist sand and kept in the dark at 28°C and 50% relative humidity (RH). Emerged adult SHBs were removed from the pupation containers and sexed (Neumann et al., 2001a). Males and females were kept in separate containers, equipped with feeders providing water, 50% sucrose solution and pollen patties *ad libitum*, at 34°C and 60% RH until they reached sexual maturity (∼21 days). To ensure that the SHBs were in the same physiological state, beetles were starved for 1 week prior to the start of the behavioural and trophallactic interaction assays.

Honeybees

Brood frames from six queenright *A. m. scutellata* colonies were collected from the University of Pretoria's apiary and incubated at 35°C and 50% RH in darkness to simulate in-hive conditions. One hundred newly emerged bees (≤24 h old) were collected from each frame and marked on the thorax using a paint marker (Schneider, Germany) and reintroduced back into their respective hives. After 6 days, 40 of the marked bees (6- to 7-day-old nurse bees by now), together with 20 foragers were collected from each colony. Pollen and nectar/water foragers were collected at the hive entrances as they returned from foraging. Twenty-four hours prior to collecting the marked nurse bees and foragers and commencing the behavioural assays, brood frames (ready to emerge) were collected from the same hives at the apiary and incubated as described above. Newly emerged bees were collected within 24 h for the behavioural assays.

Behavioural assays

Behavioural interactions

Behavioural observations were made using disposable Petri dishes (100×15 mm) as observation arenas. A trial consisted of placing five sexually matured SHBs of the same sex, a newly emerged, a 6- to 7-day-old nurse and a forager bee in the observation arena and recording their behaviour for 2 h, using a camcorder with Exmor R™ and SteadyShot™ (Sony, Tokyo, Japan). A total of 48 trials were recorded (four trials using female SHBs and four trials using male SHB for each of six honeybee colonies). Recorded videos were analysed and the type and duration of each observed interaction were noted (see Table 1 for a description of the interactions). For each interaction, the occurrence and duration were determined, with the exception of stinging (an instantaneous behaviour) and shoving interactions, for which only occurrence data were noted.

Determination of protein transfer during SHB–bee trophallactic interactions

To determine whether protein-rich secretions from the hypopharyngeal glands are transferred to SHBs during trophallactic interactions, we injected nurse bees with ^{14}C-labelled phenylalanine (L-[^{14}C(U)]-phenylalanine, >360 mCi mmol^{-1} 0.1 mCi ml^{-1}; PerkinElmer, Waltham, MA, USA) as a qualitative marker for protein transfer to SHBs according to the methods described by Crailsheim (1990, 1992) for measuring protein transfer. To account for any ^{14}C-phenylalanine that could have been transferred to the SHBs through ingestion of haemolymph from gnawing on the bees, ^{3}H-labelled polyethylene glycol ([1,2-^{3}H]-polyethylene glycol, 0.05–2 mCi g^{-1}, PerkinElmer) was used as a marker for ingested bee haemolymph. Polyethylene glycol is a suitable haemolymph marker because it is not absorbed by the intestinal tract nor is it metabolised (Crailsheim, 1985).

Radioactive labelling

Six- to 7-day-old nurse bees were immobilised on ice and secured to a piece of wood using two insect pins crossed between the thorax and abdomen. While in this position, the bees were injected with 1 µl of a solution of ^{3}H-polyethylene glycol:^{14}C-phenylalanine in a

Table 1. The different types of behaviour accounted for during the recorded video analysis

Individuals	Behavioural interaction	Description of behaviour
Bee–bee	Antennating	Any form of antennal contact with a bee, including investigating and any contact preceding being fed by a bee through trophallaxis (solicitation/begging behaviour)
	Trophallaxis/trophallactic interaction	Defined as trophallactic contact during which a drop of food is transferred following successful solicitation
SHB–bee	Antennating	Any form of antennal contact with a bee, including investigating and any contact preceding being fed by a bee through trophallaxis (solicitation/begging behaviour)
	Trophallaxis/trophallactic interaction	Defined as trophallactic contact during which a drop of food is transferred following successful solicitation
	Mounting	SHB mounts bee abdomen and cuts tissue between tergites using mandibles
	Predation	SHB feeding on a honeybee worker
	Stinging	Bee attempts to sting SHB
SHB–SHB	Antennating	Antennal contact with one or more SHB
	Shoving/interference	During SHB–bee trophallactic interactions, a second interfering SHB pushes or shoves the initiating SHB away to get the reward

SHB, small hive beetle.

ratio of 3:2 (or 1.5:1) (containing a total 0.05 µCi), between the fifth and sixth abdominal segments using a microsyringe (5 µl Microliter Syringe Model 65 RN, Hamilton, USA). If haemolymph oozed out after the needle was removed, it was lightly blotted with a strip of filter paper (referred to as the 'checking filter paper' and used to determine the amount of radioactivity successfully injected). If the volume of haemolymph lost was more than an estimated 1 µl, the bee was not included in the experiment. The injection wound was then sealed with a drop of warm beeswax mixed with collophonium. Bees were kept in this position for 2 h in room conditions. To confirm the distribution of the radioisotopes in body compartments, control bees were collected immediately after injection, as well as 2 h post injection. To control for any injection volume inconsistencies owing to the small injection volume used, 1 µl of injection solution was injected onto a strip of filter paper (referred to as the 'reference filter paper') before each injection and used to determine the injection volume and radioactivity injected.

Trophallactic interaction assays

Two hours post injection, bees were carefully removed and placed in individual disposable Petri dishes (the observation arena) to which five same-sex SHBs were introduced. The trial started the moment the SHBs were introduced, and once a trophallactic interaction between an SHB and a honeybee had occurred, the trial was stopped, and the bee and SHBs were collected and frozen at −20°C until further analysis. In total, 50 trials were conducted (27 female and 23 male), but only samples where at least one trophallactic event had been observed were included in the analysis (100 SHB in total).

Determination of the radioactivity

The collected bee samples were dissected into head, thorax and abdomen, and homogenised separately in 200 µl 80% ethanol before addition of 1 ml Soluene®-350 (PerkinElmer) and subsequent incubation for 48 h at room temperature. Total radioactivity was counted in each sample using a Tri-Carb 2800 TR liquid scintillation counter (Packard, Downeres Grove, IL, USA) and 4 ml of ULTIMA Gold™ XR scintillation fluid (PerkinElmer) per sample. The lower and upper limits of the energy windows used for ^{3}H and ^{14}C were 0 and 12 keV and 12 and 156 keV, respectively. To count the total radioactivity on the reference and checking filter paper strips, the filter paper strips were placed in 500 µl 80% ethanol and incubated for 2 h before adding 4 ml of scintillation fluid. Recovery rates (determined using control bee samples) were calculated using the total amount of radioactivity injected per bee. The total amount of radioactivity injected was calculated as follows: total radioactivity=amount of radioactivity injected–amount of radioactivity lost. The amount of radioactivity injected was determined using the reference filter paper strips; the amount of radioactivity lost (the radioactivity lost by haemolymph pressing out after the injection) was determined using the checking filter paper strips.

The presence of ^{14}C in SHBs was considered a positive result for protein transfer from bee to SHB during trophallactic interactions. To correct for any ^{14}C that could have been transferred to the SHB through ingestion of haemolymph, the ^{3}H:^{14}C ratio for each SHB sample was determined and compared with the expected ^{3}H:^{14}C ratio in the haemolymph (validated using the reference filter paper strips and the control bee samples). Significant deviations from the average expected ^{3}H:^{14}C ratio in the haemolymph in favour of higher ^{14}C levels were considered indicative of ^{14}C transfer through trophallaxis in addition to the ^{14}C obtained through gnawing and licking.

Statistical analyses

All data were evaluated for normality and homogeneity of variance prior to analysis. A general linear model and *post hoc* Bonferroni tests were performed to determine whether specific interactions occurred more frequently than others and whether the average duration of these differed. Kruskal–Wallis ANOVA (KWA) was used to determine the effect of SHB sex on the type, occurrence and duration of the interactions. Chi square tests were used to evaluate the effect of the age of the bees on the type, occurrence and duration of the interactions between the bees and SHBs. The alpha level was set to 0.05 for all analyses. All analyses were performed using STATISTICA v13.2 (TIBCO Software Inc., Palo Alto, CA, USA).

RESULTS

Behavioural assays

A total of 2360 behavioural interactions were observed from 48 trials conducted. Of these behavioural interactions, 77% were antennating and trophallactic events. Most of the observed antennating and trophallactic events were bee–bee interactions (66%), followed by SHB–bee interactions (20%) and SHB–SHB interactions (14%). Male SHBs interacted and antennated with all three age cohorts of bees, but trophallactic interactions were limited to newly emerged and nurse bees (Fig. 1, Table 2). Female SHBs, in contrast, antennated and solicited food from all three age cohorts of bees (Figs 1 and 2).

Journal of Experimental Biology (2021) 224, jeb234807. doi:10.1242/jeb.234807

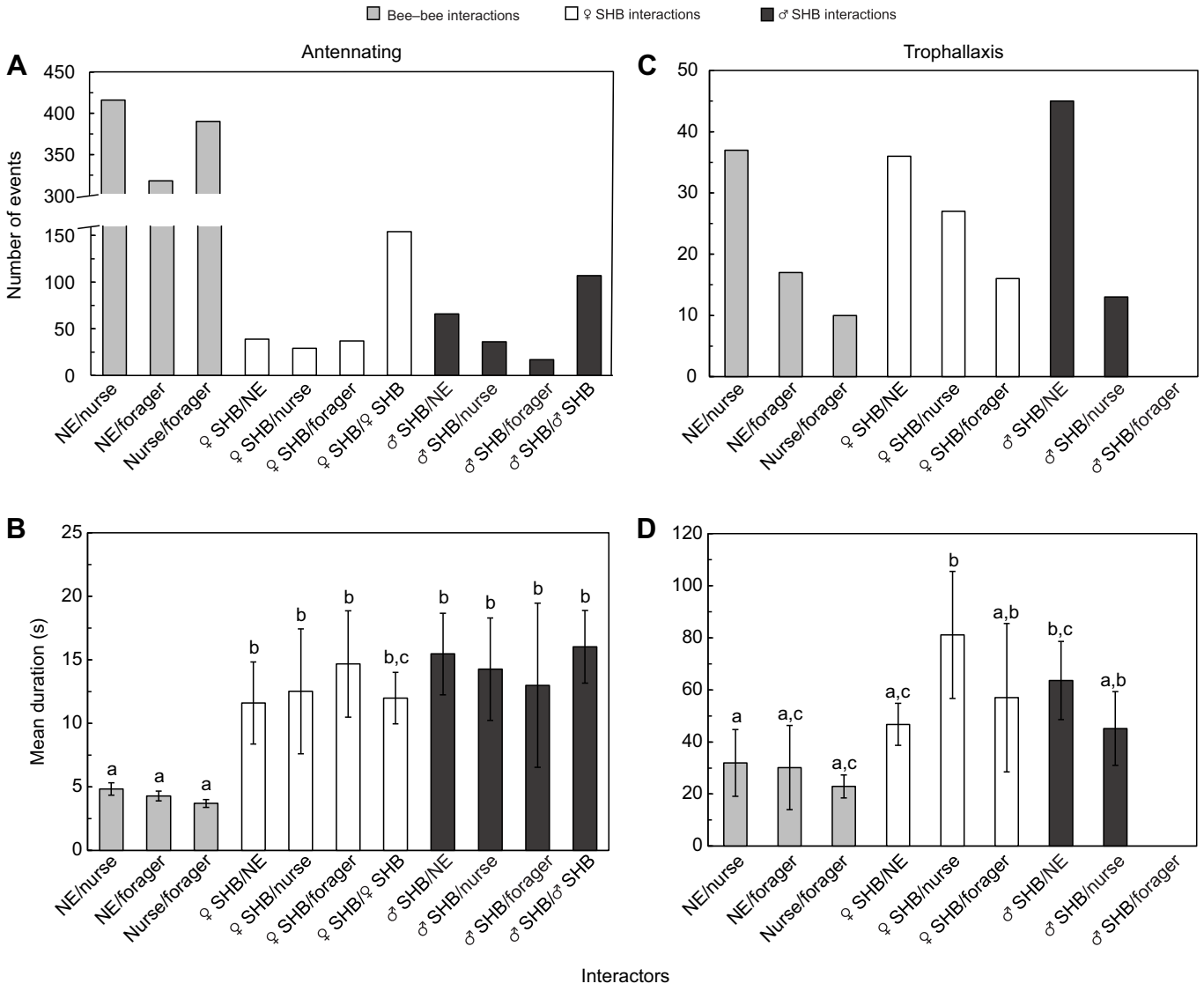


Fig. 1. Antennating and trophallactic events observed between small hive beetles (SHBs) and different age cohorts of worker bees. The (A) number of antennating events and (B) the durations of antennating (indicated as means±s.e.m.) events between ♀ SHBs and bees were not significantly influenced by the age of the bees (χ^2=1.5999, d.f.=2, P>0.05). The number of ♂ SHB–bee antennating events were significantly influenced by the age of the bees (χ^2=30.7747, d.f.=2; P<0.05). Interactor identity had no significant effect on the mean antennating duration (KWA: $H_{3,485}$=1.430620, P>0.05), with ♂ SHB–♂ SHB antennating interactions significantly longer than ♀ SHB–♀ SHB antennating interactions (KWA: $H_{1,261}$=6.121323, P<0.05). (C) The number of trophallactic interactions were significantly influenced by the age of the bee (♂ SHB–bee χ^2=55.4836, d.f.=2, P<0.05; ♀ SHB–bee χ^2=7.6203, d.f.=2, P<0.05). (D) The duration of trophallactic interactions (indicated as means±s.e.m.). The longest trophallactic interactions occurred between ♀ SHBs and nurse bees and the shortest occurred between ♂ SHBs and nurse bees. Means denoted by a different letter indicate significant differences between interactors (P<0.05). NE, newly emerged bee; nurse, nurse bee.

SHB antennating interactions

The age of the bees had no significant effect (χ^2=1.5999, d.f.=2, P>0.05) on the number or duration of the antennating events between female SHBs and bees (Fig. 1). In contrast, the number of antennating events between male SHBs and bees was significantly influenced by the age of the bees, but not the duration of the antennating events (χ^2=30.7747, d.f.=2, P<0.05) (Fig. 1). Antennating interactions between male SHBs and newly emerged bees had the highest occurrence and persisted for the longest periods of time (mean duration of 15.46±13.36 s), but the mean durations of male SHB–newly emerged bee antennating events were not significantly longer than the mean durations of male SHB interactions with the other age cohorts of bees (Table 2, Fig. 1B). The longest SHB–bee antennating duration was recorded between a female SHB and a forager and lasted for 60.11 s, though the mean duration of female SHB–forager antennating events was 14.68±2.13 and interactor identity had no significant effect on the mean antennating duration when comparing bee age and sex of SHBs (KWA: $H_{3,485}$=1.430620, P>0.05) (Table 2, Fig. 1). Male–male SHB antennating interactions were significantly longer than female–female SHB antennating interactions (KWA: $H_{1,261}$=6.121323, P<0.05).

SHB trophallactic interactions

When comparing the mean duration of antennating and trophallactic events across all of the interacting groups, antennating events were significantly shorter than trophallactic events (KWA: $H_{1,1810}$=436.3169, P<0.05). The longest

Journal of Experimental Biology (2021) 224, jeb234807. doi:10.1242/jeb.234807

Table 2. Number and duration of antennating and trophallactic interactions observed in the behaviour assays

Behaviour	Interactors	Number of interactions	Mean±s.e.m. (s)	Minimum (s)	Maximum (s)
Trophallaxis	NE+nurse	37	31.90±6.55	5.74	229.38
	NE+forager	17	30.15±8.24	7.49	129.22
	Nurse+forager	10	22.96±2.26	14.34	34.55
	♀ SHB+NE	36	46.78±4.12	9.94	117.40
	♀ SHB+nurse	27	81.15±12.44	13.70	245.68
	♀ SHB+forager	16	57.00±14.55	5.16	256.25
	♂ SHB+NE	45	63.63±7.64	8.10	305.28
	♂ SHB +nurse	13	45.18±7.21	16.42	95.86
Antennating	NE+nurse	416	4.83±0.25	0.31	51.80
	NE+forager	318	4.28±0.2	0.25	24.32
	Nurse+forager	390	3.69±0.15	0.30	25.71
	♀ SHB+NE	39	11.61±1.64	2.90	46.38
	♀ SHB+nurse	29	12.52±2.51	1.70	54.84
	♀ SHB+forager	37	14.68±2.13	0.057	60.11
	♀ SHB+♀ SHB	154	11.99±1.03	0.31	100.058
	♂ SHB+NE	66	15.46±1.64	1.44	55.20
	♂ SHB+nurse	36	14.27±2.05	2.64	55.86
	♂ SHB+forager	17	13.00±3.29	2.43	49.80
	♂ SHB+♂ SHB	107	16.03±1.46	1.30	68.10

Each of the 48 SHB same-sex trials consisted of 5 ♀ SHBs or ♂ SHBs, 1 NE, 1 nurse and 1 forager. NE, newly emerged bee; nurse, nurse bee; SHB, small hive beetle.

trophallactic interactions occurred between female SHBs and nurse bees and lasted 81.15±64.66 s (n=27) (see Fig. 1, Table 2), while the shortest trophallactic interactions occurred between male SHBs and nurse bees with a mean duration of 45.18±26.01 s (n=13). There were no significant differences in the duration (KWA: $H_{1,\ 81}$=1.745367, P>0.05) of male SHB–newly emerged bee (63.63±51.26 s; n=45) and female SHB–newly emerged bee (46.78±24.72 s; n=36) trophallactic interactions.

The age of the bees had a significant influence on the number of trophallactic events female SHBs (χ^2=7.6203, d.f.=2, P<0.05) and male SHBs (χ^2=55.4836, d.f.=2, P<0.05) engaged in with the bees (Fig. 1). The highest occurrence of trophallactic events were observed between male SHBs and newly emerged bees (n=45), followed by female SHB–newly emerged bee trophallactic interactions (n=36) (Fig. 1, Table 2). No trophallactic interactions were observed between male SHBs and forager bees, but at least 16 observations were made of female SHBs soliciting food from forager bees with a mean duration of 57.00±58.19 s (Table 2).

SHB and honeybee aggression

The most aggressive behaviour displayed by the SHBs was that female SHBs mounted newly emerged bees more often than did male SHBs (Fig. 3A), but there were no significant differences in the mean duration of these interactions (KWA: $H_{1,58}$=1.990573, P>0.05). Nurse bees (n=9) were mounted less often by SHBs than newly emerged (n=58) and forager bees (n=32) (Fig. 3A). Male (n=26) and female (n=28) SHBs predated on forager bees with similar frequencies, whereas the newly emerged and nurse bees managed to escape predation from male SHBs, with only a couple of female SHBs (n=2) managing to predate on nurse bees (Fig. 3A) (see Table S1 for the number and duration of mounting and predation interactions). We also observed ‘interference’ or ‘shoving’ events during SHB–bee trophallactic interactions in 41% of the trials (Fig. 3A). This involves a second interfering SHB pushing or shoving the initiating SHB away to obtain the reward.

Honeybees displayed similar levels of aggression towards male and female SHBs with no significant difference between

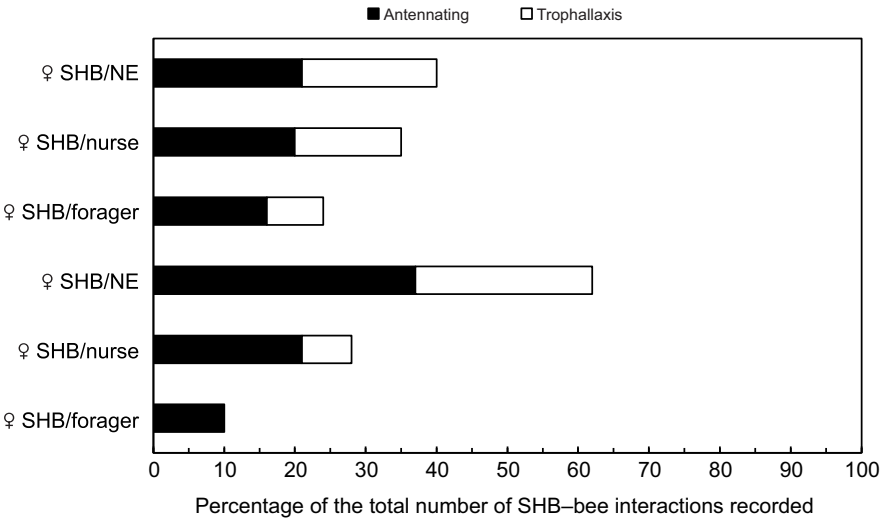
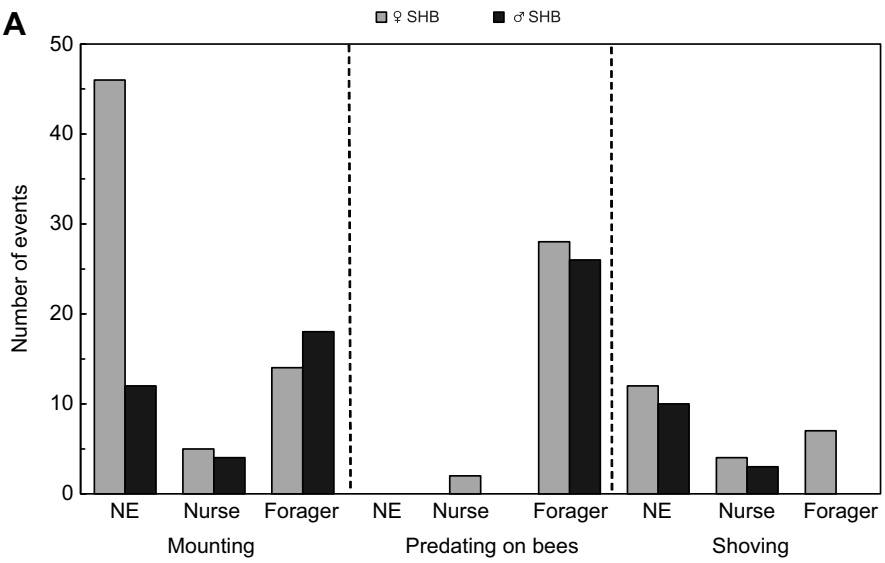


Fig. 2. A comparison of the interactions (antennating and trophallaxis) that transpired between SHBs and each age cohort of bees grouped by SHB sex. The relative percentage of antennating and trophallactic events is also indicated. Male SHBs interacted and antennated with all three age cohorts, but trophallactic interactions were limited to newly emerged and nurse bees. Female SHBs antennated and engaged in trophallactic interactions with all three age cohorts of bees. The two sexes engaged in a similar number of SHB–bee interactions.

Journal of Experimental Biology (2021) 224, jeb234807. doi:10.1242/jeb.234807



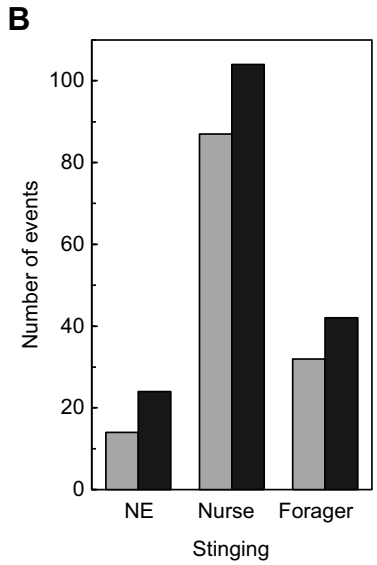


Fig. 3. Aggressive behaviours displayed by SHB and honeybees. (A) SHBs shoved each other while aggression towards the worker bees included mounting and predation on the bees. (B) Honeybees displayed similar levels of aggression towards ♂ SHBs and ♀ SHBs. There was no significant difference between the number of sting attempts on ♂ SHBs and ♀ SHBs (χ^2=4.5366; d.f.=2, P>0.05). Nurse bees had the highest recorded occurrences of sting attempts on SHBs.

the number of sting attempts on male or female SHBs (χ^2=0.9927, d.f.=2, P>0.05). The age of the bees had a significant effect on sting attempts overall (χ^2=126.713, d.f.=2, P<0.05). Nurse bees had the highest recorded occurrences of sting attempts on female and male SHBs, with 87 and 104, respectively. Forager bees made 32 and 42 sting attempts on female and male SHBs, respectively. Newly emerged bees showed the least aggression towards SHBs and only attempted to sting female and male SHBs 14 and 24 times, respectively (Fig. 3B).

Honeybee antennating and trophallactic interactions

Bee to bee antennating events amongst different age cohorts were shorter in duration (3.68–4.82 s) than antennating events between honeybees and SHBs (11.61–16.03 s) (Fig. 1, Table 2). Intra-species antennating, for both SHBs and honeybees, were more frequent than inter-species antennating (SHB–honeybee) (Fig. 1). The mean duration of bee–bee trophallactic interactions was 30.04 s (n=64, 95% CI: 21.48, 38.59), whereas the mean duration of SHB–bee trophallactic interactions was 60.13 s (n=137, 95% CI: 51.95, 68.31).

Protein transfer during SHB–bee trophallactic interactions

The average radioactivity injected per bee was 0.051±0.007 µCi, with an average ^{3}H:^{14}C ratio of 58±3:42±3. The average rate of recovery of radioactivity was 88% and 92% for ^{3}H and ^{14}C, respectively. A total of 100 SHBs (65 females and 35 males) were analysed for the presence of ^{14}C based on visual observations of trophallactic interactions. ^{14}C were detected in 39 (39%) of these SHBs, demonstrating that protein or proteinaceous secretions from the hypopharyngeal glands were transferred to SHBs during trophallaxis (Table 3). In 10 of these SHBs, ^{3}H was also detected, indicating that these SHBs had gnawed on the bees prior and possibly after the trophallactic interactions occurred (prior to trophallactic interactions gnawing was observed). Of the 65 female SHBs analysed after visual observations of trophallaxis, ^{14}C was present in 28 (43%) of the females. Of the 35 male SHBs analysed after visual confirmation of trophallaxis, ^{14}C was present in 11 (31%) of the males. SHB sex significantly affected the likelihood that protein was transferred during SHB–bee trophallactic interactions, (χ^2=6.329, d.f.=1, P<0.05) (Table 3).

Interestingly, in several of the of the trials, more than one SHB tested positive for the presence of ^{14}C in the same trial indicating

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Table 3. The transfer of ^{14}C-phenylalanine from nurse bees to SHBs

SHB Sex	Number of SHBs used in the trophallactic protein transfer assays	Number of SHBs analysed for the presence of ^{14}C after visual confirmation of trophallaxis	Number of SHBs that tested positive for the presence of ^{14}C
Female	135	65 (48%)	28 (43%)
Male	115	35 (30%)	11 (31%)

The presence of ^{14}C in SHBs was considered a positive result for protein transfer from bee to SHB during the observed trophallactic interactions.

multiple trophallactic events, even though the trial was terminated after a SHB–bee trophallactic interaction was observed. This could be indicative of secondary or horizontal food transfer between SHBs after successful trophallactic interactions with nurse bees.

DISCUSSION

Here, we show that in addition to worker bees sharing their crop contents with SHBs, as demonstrated by Ellis et al. (2002), worker bees also transfer protein-rich glandular secretions (jelly) to SHBs during bee–beetle trophallactic interactions. As expected, female SHBs are significantly more successful than their male counterparts in inducing nurse bees to transfer protein-rich jelly during trophallaxis. In the protein transfer assays, 48% of female SHBs engaged in trophallactic interactions with nurse bees, of which almost half received protein, compared with the 30% of male SHBs that engaged in trophallactic interactions and less than a third receiving protein. The behavioural assays mirrored the results from the protein transfer assays: 43% of female SHB–bee interactions were trophallactic, while only 33% of male SHB–bee interactions were trophallactic (Fig. 2). This is consistent with previous findings that bee-naive female SHBs are superior in trophallactic solicitation compared with bee-naive males, reflecting the female's higher nutritional requirements and drive to obtain food owing to egg production and often larger body size (Neumann et al., 2015; Pirk and Neumann, 2013). As of yet, it is not known whether nurse bees transfer both jelly and their crop contents during a single trophallactic interaction with SHBs or whether they only share either jelly or crop contents. Therefore, it is possible that female and male SHBs that engaged in trophallactic interactions but did not receive jelly could have received carbohydrate-rich crop contents instead. Ellis et al. (2002) found that 40% of SHBs (unknown sex ratio) successfully obtained sugar (crop contents) from bees of unknown age after 24 h when SHBs were introduced in a nucleus hive (the SHBs were separated with gauze from the bees, which prevented mingling but allowed antennal mouthpart contact). Unfortunately, it is not plausible to compare the success rate of obtaining jelly in this study directly with the success rate of SHB obtaining sugar in the study of Ellis et al. (2002) owing to fundamental differences in the experimental design as mentioned above. Both protein and carbohydrate are vital macronutrients in an insect's diet that provide essential amino acids and energy that influence the insect's survival, growth and fecundity (Behmer, 2009). SHBs are the only species known to mimic honeybee trophallaxis and successfully coerce worker bees to share carbohydrates and a limiting resource such as protein, essential for the bee colony's own survival and reproduction.

Curiously, we found potential evidence of secondary or horizontal food transfer between SHBs after successful trophallactic interactions with nurse bees. More than one SHB had levels of ^{14}C-phenylalanine indicative of protein transfer in more than half of the protein transfer trials or assays, yet a trial was immediately terminated as soon as a single SHB–bee trophallactic interaction had occurred. This suggests that the SHB involved in the observed SHB–bee trophallactic interaction had transferred food to the other SHBs in the trial arena without the observer realising. Alternatively, the other SHBs in the trial arena also managed to successfully solicit food from the nurse bee without the observer noticing, likely during the assay termination step. Nevertheless, the secondary or horizontal food transfer between SHBs requires further studies to tease apart these interactions and the possible influence it may have on the success of subsequent SHB–bee trophallaxis.

The behavioural assays demonstrated that female SHBs, contrary to expectation (Neumann et al., 2015), did not preferentially approach and initiate antennating interactions with a specific age cohort (Fig. 1A), whereas the males seemed to be more selective in their interactions, avoiding the older workers and favouring the docile and inquisitive newly emerged workers. Old worker bees (foraging age) are known to be more aggressive towards SHBs than newly emerged workers (Pirk and Neumann, 2013). When the total numbers of interactions (antennating and trophallaxis) that transpired between SHBs and each age cohort of bees were compared, a clear tendency of male SHBs to avoid older and more aggressive workers emerged, even though male and female SHBs engaged in a similar number of interactions (Table 2). Sixty-two percent of male SHB–bee interactions involved newly emerged bees and only 28% and 10% involved nurse bees and foragers, respectively. Female SHBs seem to be less discriminant and interacted 41%, 35% and 24% (Fig. 2) of the time with newly emerged bees, nurse bees and foragers, respectively, despite the increased risk of injury and energetic cost associated with the repeated fast advances and retreats that occur with soliciting events involving older, more aggressive and active workers (Elzen et al., 2001; Neumann et al., 2015). SHBs have been shown to be able to detect alarm pheromones at very low concentrations (Torto et al., 2007). It is possible that male SHBs detected alarm pheromones secreted by the older bees in the trial arena and tried to avoid these individuals. However, bee alarm pheromones serves as kairomones to SHBs, thus attracting them to the food resources in a colony (Torto et al., 2007). Considering that SHBs have been shown to assess the defensiveness of worker bees and adjust their behaviour accordingly (Pirk and Neumann, 2013), it is more likely that the male SHBs avoided the older bees based on the bees' aggressive posture and behaviour. Regardless, the females' bold approach was more rewarding than the cautious approach of the males. Female SHBs were more likely to engage in SHB–bee trophallaxis than male SHBs (43% compared with 33%; see Fig. 2), supporting the postulation that female SHBs will risk more to obtain food due to higher nutritional demands driven by a larger body size and oogenesis (Neumann et al., 2015; Pirk and Neumann, 2013).

The age of the interacting worker bee significantly influenced the probability of a SHB–bee trophallactic interaction occurring. We expected nurse bees to be the most likely to feed SHBs owing to their higher nutritional status as the distributors of protein-rich jelly in the hive (Crailsheim, 1992; Free, 1957). However, both male and female SHBs were significantly more often fed by newly emerged workers than any other age cohort; presumably, the more docile and inquisitive nature of this age cohort makes them easier to exploit. The newly emerged workers likely transferred crop contents, if they transferred anything. The crop contents could contain whatever food the newly emerged workers consumed from the stored food on the brood comb (nectar, honey) or food they received through trophallactic interactions with other newly emerged workers on the

brood comb (nectar, honey) before being removed from the comb and placed in the experimental arena. In addition, a newly emerged worker could have received food (protein-rich jelly, nectar or honey) through trophallactic interactions with the forager and nurse bee in the experimental arena. Male SHBs antennated less frequently with foragers than any other age cohort of bees and, unlike female SHBs, refrained from soliciting food from this active and aggressive age cohort (Figs 1 and 2). Moreover, female SHBs engaged in twice the number of trophallactic interactions with nurse bees compared with male SHBs, even though they engaged in a similar number of antennating events with this age cohort (Table 2). Considering that nurse bees displayed the highest aggression towards SHBs (Fig. 3B), these observations reiterate the wariness of male SHBs of the older, more defensive workers, and the willingness of female SHBs to take increased risks to maximise the solicitation of food. (Nurse bees may be the most aggressive towards SHBs owing to their higher investment in terms of jelly production.)

Interestingly, the mean duration of SHB trophallactic feedings (60 s) was twice as long as bee–bee trophallactic feeding events (30 s), and it was also longer than the average reported feeding durations of drones (42 s) and queens (44 s) in the breeding season (Allen, 1960; Free, 1957; Ohtani, 1974). In addition, the longest mean feeding durations were between female SHBs and nurse bees (81 s), the age cohort of worker bees with the highest nutritional status as distributors as protein-rich jelly in the hive. Presuming that longer feedings equal higher quality feedings, SHBs may have evolved the ability to manipulate the donor bee to feed it longer, inducing a higher quality feeding. Alternatively, the longer feeding durations could simply occur because the inter-species mechanical food transfer is less efficient and takes longer.

Apart from antennating and trophallaxis, other more aggressive SHB behaviours were also observed, including shoving or interference behaviour and predation on bees. These behaviours are described and discussed in detail by Neumann et al. (2015) and Pirk and Neumann (2013).

In conclusion, the female SHBs do not appear to interact preferentially with a specific age cohort of bees when soliciting food, while male SHBs tend to be more discriminant than female SHBs in avoiding the older, more aggressive and active worker bees. Furthermore, the results demonstrate that SHBs receive protein-rich glandular secretions from their honeybee hosts during trophallactic interactions and suggest that female SHBs are more successful than male SHBs in inducing the transfer of these protein-rich secretions. It is possible that some form of chemical mimicry is involved, albeit not to disguise the SHB as a nest mate, but rather inducing or producing the right signals to induce feeding (Crailsheim, 1998), especially to induce protein transfer.

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