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RESEARCH ARTICLE

Honey bee (*Apis mellifera*) sociability and nestmate affiliation are dependent on the social environment experienced post-eclosion

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ABSTRACT

Underpinning the formation of a social group is the motivation of individuals to aggregate and interact with conspecifics, termed sociability. Here, we developed an assay, inspired by vertebrate approaches to evaluate social behaviours, to simultaneously examine the development of honey bee (*Apis mellifera*) sociability and nestmate affiliation. Focal bees were placed in a testing chamber which was separated from groups of nestmates and conspecific non-nestmates by single-layer mesh screens. Assessing how much time bees spent contacting the two mesh screens allowed us to quantify simultaneously how much bees sought proximity and interaction with other bees and their preference for nestmates over non-nestmates. Both sociability and nestmate affiliation could be detected soon after emergence as an adult. Isolation early in adult life impaired honey bee sociability but there was no evidence for a critical period for the development of the trait, as isolated bees exposed to their hive for 24 h when as old as 6 days still recovered high levels of sociability. Our data show that, even for advanced social insects, sociability is a developmental phenomenon and experience dependent.

KEY WORDS: Social insect, Group cohesion, Nestmate recognition, Isolation, Aggregation

INTRODUCTION

Foundational to any social group is a motivation of its members to aggregate and interact with each other, referred to as their sociability (Ward and Webster, 2016). While often considered an instinctive behavioural response for social species, there is abundant evidence from mammalian models that the expression of sociability is sensitive to early life experience. Classic studies with rhesus macaques (*Macaca mulatta*) have shown that isolation early in life abolishes normal social behaviours, impacting the ability to affiliate and live amongst peers (Harlow et al., 1965; Hinde, 1971). Similar findings have been reported in rodents (Olsson and Westlund, 2007) where social isolation early in life results in overtly aggressive Wistar rats (*Rattus norvegicus*) (Tóth et al., 2008, 2011) and socially withdrawn male and female mice (*Mus musculus*) (Bouet et al., 2011). The normal development of sociability is essential for the success of social animals. We would expect this to be especially true for the social insects, but while a lot of research has described the development and mechanisms of nestmate recognition in eusocial hymenoptera (Breed, 2003; van Zweden and d'Ettorre, 2010), experimental investigation of the development of their sociability has not yet been addressed. Here, we explored the development of honey bee (*Apis mellifera*) sociability and affiliation to nestmates.

The honey bee, an advanced social insect, lives in large societies featuring a complex organisation that depends on cooperative and altruistically motivated individuals (Michener, 1974), as well as continuous communication through frequent social interactions (Huang and Robinson, 1992). The colony consists of thousands of sterile female workers which perform all tasks needed to support the reproductive output of the single queen (Seeley, 1989). The group remains cohesive by distinguishing nestmates from non-nestmates, evidenced by the guard bees at the entrance of the hive which successfully prevent non-nestmates from entering the nest but allow free movement of nestmates (Breed et al., 1992). Classic studies have demonstrated the nature of the cues honey bees use for nestmate recognition (reviewed in Breed, 2003) and, like rodents (Kareem and Barnard, 1982; Villavicencio et al., 2009), bees primarily use olfactory cues to discriminate between group members and non-members (van Zweden and d'Ettorre, 2010). The existence of such a cohesive group also requires each individual member to be motivated to aggregate with others. Although sociability has been mentioned in terms of the social system adopted by various bee species (Fialho et al., 2014; Tomé et al., 2014; Santos et al., 2016) or indirectly studied with regards to their social interactions and communication (e.g. Huang and Robinson, 1992; Schulz et al., 2002), sociability has not been directly measured in bees.

The importance of assessing sociability separately from nestmate recognition has been demonstrated by research with rodents that sought to develop animal models for mental disorders, such as autism or depression, in which social recognition is intact but motivation to socialise and interact is impaired (Moy et al., 2004). Inspired by the methods utilised in these and other studies on social bond formation in mammals (Williams et al., 1992; Moy et al., 2004, McGraw and Young, 2017), we here developed a bioassay to assess bee sociability by quantifying how much time a focal bee spent in proximity to other bees. A group of nestmate and non-nestmate conspecifics were presented simultaneously to determine whether the focal bee preferentially affiliated with its nestmates over unfamiliar conspecifics. Although successful discrimination between nestmates and non-nestmates is reduced in contexts away from the hive (Couvillon et al., 2013, 2015), generally bees are more aggressive toward unfamiliar conspecifics and preferentially aggregate with nestmates (Breed et al., 1985; Downs and Ratnieks, 1999). We would therefore expect naturally raised bees to spend more time with nestmates than non-nestmates. Both genetic and environmental olfactory cues are used by bees to recognise nestmates (Breed, 1983; Breed et al., 1985, 1988, 1998; Breed and Stiller, 1992; Downs and Ratnieks, 1999; D'Ettorre et al., 2006), and acquisition of these identity cues occurs in the first 24 h post-eclosion (Breed et al., 1995). We therefore predicted that high levels of sociability and a

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Received 26 October 2017; Accepted 13 December 2017

Journal of Experimental Biology (2018) 221, jeb173054. doi:10.1242/jeb.173054

preference to affiliate with nestmates would appear early on and remain throughout adult life in honey bees.

As in mammals, partial or temporary isolation has been reported to negatively impact hymenopteran social interactions, mortality and brain anatomy (Boulay and Lenoir, 2001; Boulay et al., 2000; Koto et al., 2015; Maleszka et al., 2009; Seid and Junge, 2016). Importantly, social contact and interactions initiate the neural changes that lead to an enduring social attachment in prairie voles (Williams et al., 1994; Winslow et al., 1993; reviewed in Young and Wang, 2004), and social encounters are also essential for honey bee development and social organisation (Huang and Robinson, 1992). We hypothesised that both honey bee sociability and nestmate affiliation would be negatively impacted by total isolation from eclosion onwards. Correct development of social behaviour in vertebrates commonly occurs when young animals are exposed to an appropriate social environment within a critical time period early in development (Scott, 1962; Bouet et al., 2011; Prounis et al., 2015). Here, we explored how isolation of adult bees early in development affects sociability and nestmate affiliation and whether there is a critical period for the development of either trait.

MATERIALS AND METHODS

The arena

The well-established protocol for sociability and social recognition in rodents (Moy et al., 2004) inspired the design of the arena developed for this study (Fig. 1). The arena was manufactured by Allplastics Engineering Pty Ltd (Sydney, Australia). It consisted of three white Perspex chambers. The larger central chamber contained the focal bee and was flanked by two separate side chambers. When the side chambers' sliding sides were pulled out, the central chamber was separated from the side chambers by a soft mesh screen only (mesh spacing 1.5 mm). One side chamber contained 5–6 nestmates (NM) and the other side chamber contained the same number of non-nestmate conspecifics (NON). All sensory stimuli could pass through the mesh, and when the focal bee was located on the mesh sides, antennal or proboscis contact could occur between the stimulus bees and focal bee. Side chambers were easily switched between each test to rule out any side bias. The tops of all three chambers were made of transparent Perspex, allowing video recording for later data extraction by an observer blind to the treatment of the focal bee and the side on which nestmates were located.

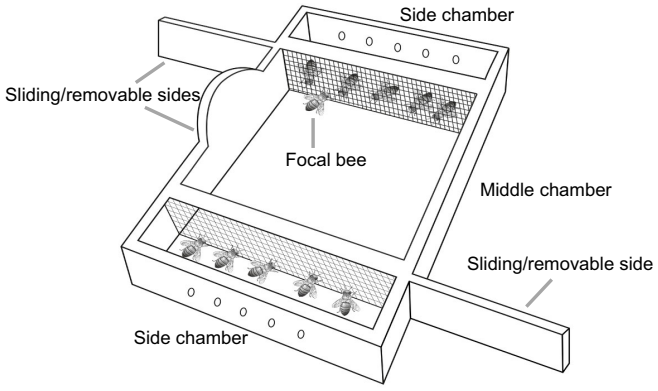

Fig. 1. Schematic diagram of the bioassay arena developed to test honey bee sociability and nestmate affiliation. The two side chambers (6 cm×2 cm×2 cm) align with the two mesh sides of the middle chamber (6 cm×6 cm×2 cm). They can be sealed by sliding in the removable side, allowing any bees inside to be caught and transported without anaesthetising. Small holes on the sides allow bees to be fed with honey. The middle chamber also has a sliding side so focal bees can be easily placed inside and removed after the trial. All top sides of the chambers are made of transparent Perspex so that the observer can video record the trial and score interactions live. Trials began when the sliding sides were pulled back, leaving only the mesh layer between the chambers.

Test subjects

All honey bee (*Apis mellifera* Linnaeus 1761) colonies were maintained at Macquarie University Fauna Park in Marsfield, Sydney, Australia. Honey bees were sourced from three colonies and a total of 1344 individuals were tested. To isolate bees with no social experience as adults, we collected adult bees as they emerged from their wax pupal cell. When bees began nibbling the wax cap of their pupal cell away from the inside, they were gently removed from their cell using soft tweezers. Isolated bees were then placed individually in a 50 ml Falcon tube and held in a dark incubator at 33°C and 65% humidity and fed 40% sucrose until the day of testing. Crucially, isolated bees did not interact with another bee as their cells were capped during the larval stage. To obtain hive-reared bees of known age, newly emerged bees were painted (Uniball POSCART, Mitsubishi Pencil Co. UK) on the dorsal thorax and returned to their natal hive.

Experiment 1 ran during summer 2015–16 and tested the effects of different periods of isolation on honey bee sociability and nestmate affiliation. Hive-reared bees were tested at ages 1–7, 10 and 20 days old and isolated bees were tested at age 0 (within 2 h of emergence) and 1–5 days old. Young bees are tasked with caring and feeding larvae, and must ingest pollen as well as honey to make larval food rich in protein and carbohydrates (Crailsheim and Stolberg, 1989). Hence, protein metabolism is greater and the hypopharangeal glands enlarged in young bees compared with older foragers (Huang et al., 1994). Crailsheim and Stolberg (1989) examined in detail the impact of diet on the proteolytic activity and hypopharyngeal gland size of bees reared in isolation and showed that isolation accelerated changes in young bees in all diet conditions, but sucrose-only and honey-only diets had the severest effect. To test for any dietary effects on the behaviour of isolated bees in this study, a separate treatment group of bees was isolated until 5 days of age and fed honey only or a honey–pollen paste mix (Crailsheim and Stolberg, 1989; Ament et al., 2011), *ad libitum*.

We needed to establish whether normally raised bees reacted to the presence of bees in the side chambers behind the mesh screens. To test whether time spent on the mesh screens was influenced by the presence of stimulus bees in the side chambers, we compared the time spent on the mesh by hive-reared bees in social tests (NM and NON in the side chambers) and control tests (both side chambers empty).

Experiment 2 ran during summer 2016–17 to determine whether a critical period existed for honey bee sociability and nestmate affiliation. Bees were isolated on eclosion (as above) until the age of testing, or returned to their natal hive at various time points before testing (Table 1). For example, the 3di_5 group was isolated when eclosing and returned to its natal colony at 3 days of age until testing at 5 days of age. If a critical period exists before 5 days, we would expect to see sociability scores and nestmate discrimination abilities similar to those of bees raised in their natal hive for the full 5 days (5d_5), as social experience during the critical period would recover these behaviours. During isolation, bees were fed 40% honey water. Bees that were returned to their natal hive after 2 days of age were placed into the hive on an empty frame sealed entirely with a single

Journal of Experimental Biology (2018) 221, jeb173054. doi:10.1242/jeb.173054

Table 1. Details of each treatment in experiment 2 and each group ID code

Age isolated (days)	Age returned to natal hive (days)	Age tested (days)	I.D.
–	0	5	5d_5
0	1	5	1di_5
0	2	5	2di_5
0	3	5	3di_5
0	4	5	4di_5
0	–	5	5di_5
0	5	6	5di_6
0	–	6	6di_6
0	6	7	6di_7
0	–	7	7di_7

mesh layer. This prevented the focal bees from potentially being removed by their sisters, but allowed them to interact and experience the hive environment.

Pre-experimental procedure

On the morning of testing, bees were collected individually in Falcon tubes from their colony (experiment 1 – hive-reared bees, experiment 2 – bees that spent 16 h or more in their natal colony prior to testing) 20–45 min before testing began. Isolated bees were taken from the incubator 15 min before testing. If more than 10 bees were collected (maximum 20), bees to be tested after number nine were provided with sucrose solution in their holding tubes. A randomised testing order was used for each testing bout. The testing room was heated to 25°C and lights were kept on. NM and NON stimulus groups were collected simultaneously with the first collection of focal bees from the hive, and caught directly from the top of a frame in the lower brood box into a side chamber. All stimulus bees were randomly selected from the frame but any bees visibly newly emerged were not used. Between testing bouts (30–90 min), stimulus groups were fed honey in their side chamber to reduce their motivation to beg from focal bees. A maximum of two testing bouts were run per day and the same stimulus bees were used for the entire day then killed by freezing.

Experimental procedure

A focal bee was transferred from its holding tube into the central chamber and allowed to acclimate for approximately 1 min with all sliding sides closed (Fig. 1). To begin the 5 min test, the sliding sides of the side chamber were removed and only the mesh remained between the stimulus bees and the focal bee. The time spent by the focal bee on each mesh side, the number of visits to both mesh sides and whether any dyadic interactions occurred were recorded. Dyadic interactions included reciprocal and rapid antennal contact between the focal bee and a stimulus bee for greater than 2 s and/or extension of the proboscis for trophallaxis.

Any test bees that expressed a visibly abnormal walking gait were removed from the data set. Once testing was complete, all animals were killed by freezing. All chambers were washed with 80% ethanol, rinsed with cold water and dried before reuse.

Measurements and data analysis

All statistical analyses were done in R version 3.2.0 and confidence limits were set at 95% unless otherwise stated. To minimise observer bias, data extraction from video footage was done blind; however, behavioural and dyadic interaction scoring was done live and was not blind.

Experiment 1a: testing the responses of bees in the new behavioural assay

To validate that focal bees reacted to the presence of bees in the side chambers behind the mesh screens, we compared the time spent on the mesh by hive-reared bees in social tests (NM and NON in the side chambers) and control tests (both side chambers empty). As data were not normally distributed, a Kruskal–Wallis test was used, followed by Dunn's *post hoc* test (Holm–Bonferroni method). Isolated bees were also assigned to either a social or control test, and were expected to spend comparable amounts of time on the mesh sides in both tests. To compare all four rearing groups (social–hive-reared, control–hive-reared, social–isolated and control–isolated), data were pooled from ages 1–5 days old and a Kruskal–Wallis test used followed by Dunn's *post hoc* analysis.

Similarly, we used a Kruskal–Wallis test to compare time spent on the mesh by 5-day-old isolated bees fed sucrose only, honey only or a honey and pollen mix to rule out dietary effects.

Experiment 1b: development of sociability and nestmate affiliation

Sociability was measured as the amount of time spent on the two mesh sides during the 5 min (300 s) test. Nestmate affiliation was measured as the proportion of time spent on the NM mesh out of the total time spent on the two mesh sides. We compared scores of hive-reared and isolated bees of the same age, up to 5 days old, in social tests with conspecifics present. As data were not normally distributed in both cases, a Kruskal–Wallis test followed by Dunn's *post hoc* test (Holm–Bonferroni method) was used. In addition, we recorded whether a dyadic interaction occurred with a NM, a NON, both or not at all and used a *G*-test of independence to determine whether rearing altered the likelihood of a dyadic interaction, followed by pairwise comparisons with correction for multiple testing.

Experiment 2: testing for a critical period for the development of sociability in honey bees

Based on the results in experiment 1, we exposed isolated honey bees at various ages to their natal hive (Table 1) to determine whether there was a critical age after which exposure to their natal hive environment could not reinstate natural levels of sociability and nestmate affiliation. The same measures were calculated and the same statistical tests used to compare the different treatment groups as in experiment 1b.

RESULTS

Experiment 1a: honey bee responses to social stimuli in the bioassay

Overall, hive-reared honey bees spent significantly more time on the mesh sides in social tests than in control tests (Kruskal–Wallis: χ^2=160.25, d.f.=17, P<0.0001; Fig. 2A), confirming that these normally raised focal bees responded to the presence of conspecifics. Isolated bees spent a similar amount of time on the mesh sides in both social and control tests (Kruskal–Wallis: χ^2=200.06, d.f.=3, P<0.0001; Fig. 2B; Dunn's *post hoc*: social–isolated versus control–isolated, P=0.24), indicating that isolated bees did not respond to the presence of social stimuli behind the mesh. Further, the time spent on the mesh by isolated bees was comparable to that spent by hive-reared bees in control tests but significantly less than that spent by hive-reared bees in social tests (*post hoc*: social–hive-reared versus social-isolated, P<0.0001; control–hive-reared versus control–isolated, P=0.18; Fig. 2B).

There was no effect of diet on the sociability scores of isolated bees (Fig. 3). Five-day-old isolated bees fed a rich honey–pollen

Journal of Experimental Biology (2018) 221, jeb173054. doi:10.1242/jeb.173054

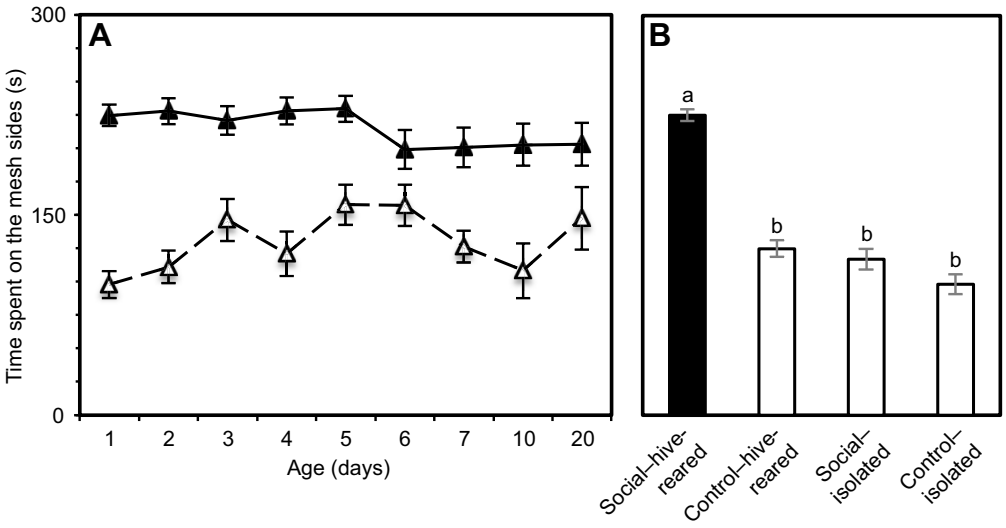

Fig. 2. New arena reliably measures honey bee sociability. (A) Hive-reared bees spent more time on the mesh in social tests (black triangles) compared with control tests (white triangles) when the side chambers were empty (Kruskal–Wallis: χ^2=160.25, d.f.=17, P<0.0001). Data points are means±s.e.m., N=832, n=19–69 (where N is the total sample size in the study and n is the sample size of each group). (B) Hive-reared bees (1–5 days old) in social tests (black bar) spent significantly more time on the mesh than hive-reared bees in control tests and all isolated bees (white bars) (Kruskal–Wallis: χ^2=200.06, d.f.=3, P<0.0001, *post hoc*: a>b, P<0.0001). Bars are means±s.e.m., N=770, n=111–289.

mix, honey only or sucrose only all had comparably low sociability scores, and all three isolated groups spent significantly less time on the mesh in proximity to conspecifics compared with 5-day-old hive-reared bees (Kruskal–Wallis: χ^2=61.53, d.f.=3, P<0.0001, *post hoc*: honey–pollen mix versus honey only, P=0.13; honey–pollen mix versus sucrose only, P=0.21; honey only versus sucrose only, P=0.26; hive-reared versus honey–pollen mix, P<0.0001; hive-reared versus honey only, P<0.0001; hive-reared versus sucrose only, P<0.0001; Fig. 3).

Experiment 1b: development of honey bee sociability and nestmate affiliation

The propensity to aggregate with conspecifics was significantly reduced in isolated bees compared with hive-reared bees and evident from 1 day of age (Kruskal–Wallis: χ^2=118.04, d.f.=10, P<0.0001; Fig. 4). All hive-reared bees aged 1–5 days old spent significantly more time on the mesh sides compared with isolated bees of the same age (Dunn's *post hoc*: P<0.01–0.0001; Fig. 4). Newly emerged bees spent an intermediate amount of time on the mesh sides, not different from that of older hive-reared or isolated bees (*post hoc*: newly emerged versus hive-reared aged 1–5 days, P=0.14–0.40; newly emerged versus isolated aged 1–3 days, P=0.35–1.00). However, progressively longer isolation, past 3 days of age, saw a further reduction in time spent in proximity to conspecifics compared with newly emerged bees (*post hoc*: newly emerged versus isolated aged 4–5 days, P≤0.01; Fig. 4). Hive-reared bees were three times as likely (76%) to have a two-way interaction with a stimulus bee than isolated bees (25%) (G=91.06, d.f.=2, P<0.0001, *post hoc*: hive-reared versus isolated, P<0.001) and this difference was also evident from 1 day of age (*post hoc*: newly emerged versus hive-reared or isolated, P≤0.01). Together, these results show that sociability is reduced at the level of both aggregation with conspecifics and social interaction with conspecifics when bees are isolated post-eclosion.

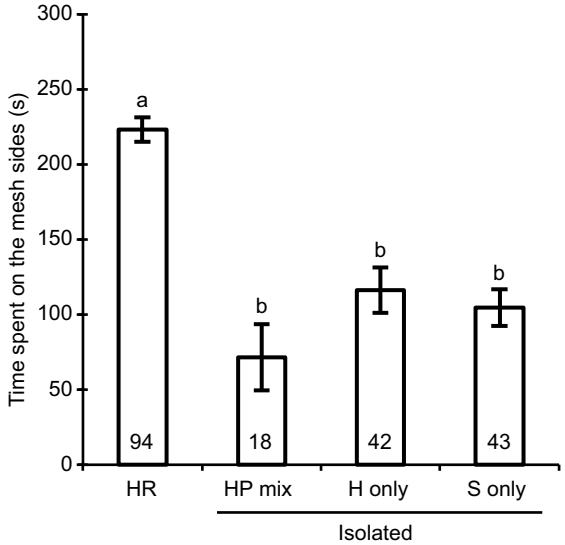

Fig. 3. No effect of diet on isolated honey bee sociability. Five-day-old hive-reared bees (HR) spent more time on the mesh compared with 5-day-old isolated bees regardless of their diet (honey–pollen mix, honey only, sucrose only) (Kruskal–Wallis: χ^2=61.53, d.f.=3, P<0.0001, *post hoc*: a>b, P<0.001).

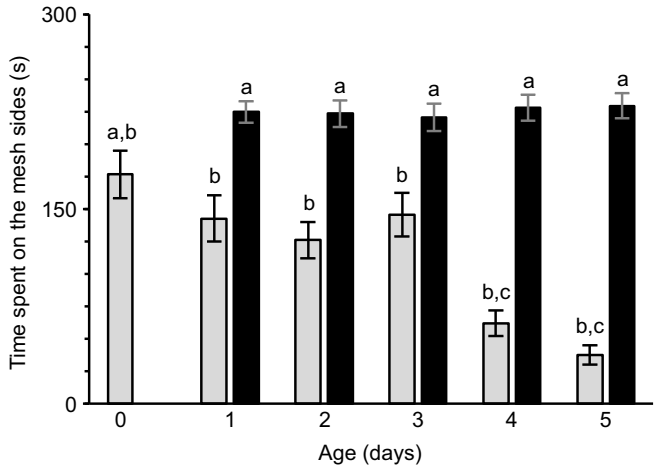

Fig. 4. Isolation from eclosion reduces levels of honey bee sociability. All hive-reared bees (black bars) aged 1–5 days old spent significantly more time on the mesh sides compared with isolated bees (white bars) aged 1–5 days old (Kruskal–Wallis: χ^2=118.04, d.f.=10, P<0.0001, *post hoc*: a>b, P<0.01–0.0001). Isolated bees aged 4 and 5 days showed significantly reduced sociability compared with initial levels recorded for newly emerged bees (*post hoc*: a,b>b,c, P≤0.01). Bars are the means±s.e., N=439, n=9–69.

Journal of Experimental Biology (2018) 221, jeb173054. doi:10.1242/jeb.173054

We next explored whether honey bees preferentially affiliated with nestmates when given the choice between bees from their natal colony (NM) and conspecifics from another unrelated colony (NON). Overall, bees raised naturally in their hive spent more time with NM than with NON (Wilcoxon signed-rank test: W=25677, N=289, P<0.001, r=−0.14; Fig. 5) but neither isolated bees (W=3539.5, N=120, P=0.57, r=−0.02; Fig. 5), nor newly emerged bees expressed preference for NM (W=275.5, N=30, P=0.10, r=−0.07). Unlike sociability, however, there was no clear development for this preference and pairwise comparisons revealed no significant differences between rearing age groups except that 5-day-old hive-reared bees expressed a greater preference for NM than did 2-day-old hive-reared bees (Kruskal–Wallis: χ^2=23.62, d.f.=10, P<0.01, *post hoc*: hive-reared 5 days old versus hive-reared 2 days old, P<0.05). In this bioassay, we found that nestmate affiliation also appeared soon after emergence but it was not detected in bees isolated post-eclosion, indicating that it is also dependent on experience of the hive environment.

Experiment 2: testing for a critical period for the development of sociability in honey bees

Honey bees were exposed to their natal hive at different ages (Table 1) to determine whether there was a time point after which experience of the hive no longer initiated natural levels of sociability. As observed in experiment 1, bees isolated until testing spent less time on the mesh sides compared with 5-day-old hive-reared bees (Kruskal–Wallis: χ^2=67.81, d.f.=9, P<0.0001, *post hoc*: isolated until 5, 6 or 7 days old versus hive-reared 5 days old, P≤0.01; Fig. 6). However, exposure to the hive environment for approximately 16 h before testing as late as 5 days of age recovered levels of sociability comparable to those of hive-reared 5-day-old bees (*post hoc*: hive-reared 5-day-old bees versus 1di_5, 2di_5, 3di_5, 4di_5, 5di_6, P=0.50–1.00; Fig. 6). Bees returned to their natal colony at 6 days old and then tested the next day (6di_7) spent an equivalent time on the mesh sides as all other treatment groups (*post hoc*: P=0.13–1.00; Fig. 6).

Next, all bees that experienced their natal hive before 6 days of age (sociable group, black bars in Fig. 6) and all bees isolated until testing (isolated group, white bars in Fig. 6) were pooled. The 6di_7 group had sociability scores similar to those of both the sociable and isolated groups (intermediates, grey bar in Fig. 6). Both the sociable group and the intermediate bees were more likely to interact with a conspecific than were the isolated bees (64%, 70%, 11.5% likelihood of an interaction, respectively, G=91.57, d.f.=2, P<0.0001, *post hoc*: sociable versus isolated, P<0.0001, 6di_7 versus isolated, P<0.0001).

Unlike in experiment 1, no preference to affiliate with NM was observed in experiment 2. There was no difference in time spent on the NM mesh between any of the treatment groups (Kruskal–Wallis: χ^2=11.25, d.f.=9, P=0.26). Furthermore, the bees with high sociability scores (sociable group) spent a similar time on the NM and NON mesh (Wilcoxon signed-rank test: V=4538, N=128, P=0.25), as did the combined isolated group (V=859, N=61, P=0.84). The intermediate bees, however, spent significantly more time on the NON mesh than the NM mesh (V=3, N=10, P≤0.01, r=−0.10).

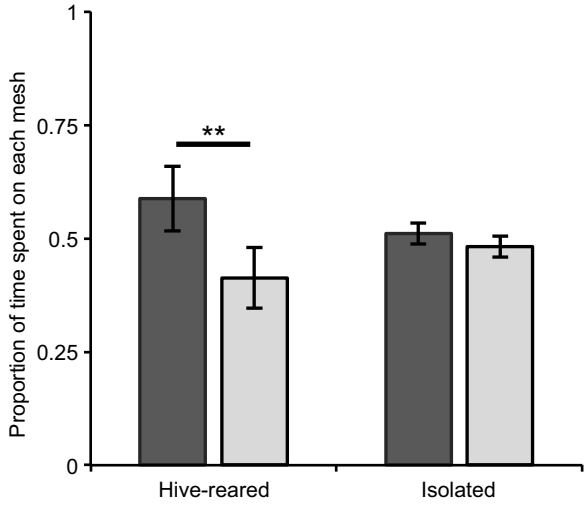

Fig. 5. Preference to affiliate with nestmates is lost in bees isolated post-eclosion. Hive-reared bees spent significantly more time on the NM mesh (dark grey bars) than the NON mesh (light grey bars) (Wilcoxon signed-rank test: W=25,677, N=289, **P<0.001, r=−0.14) but isolated bees did not express this preference for NM (W=3539.5, N=120, P=0.57, r=−0.02).

DISCUSSION

Here, we developed a bioassay to examine the development of honey bee sociability and to determine whether bees preferentially affiliate with NM over NON when given a choice. Testing of normally raised bees at different ages showed both phenomena appear early on after exposure to the hive environment, but this study also revealed that the degree of honey bee sociability and affiliation to nestmates is influenced by the nature of social experience in early adult life. Isolation reduced time spent with conspecifics and the likelihood of engaging in two-way interactions with other bees. However, exposure to their natal hive, even after 5 days of age, reinstated their sociability.

The first step was to confirm that the new assay was suitable for studying honey bee sociability. Testing bees at the same ages but in social (stimulus bees present) and control (empty side chambers) tests allowed us to determine whether the presence of bees in the side chambers altered time spent by the focal bee on the mesh. For naturally raised bees, the presence of conspecifics significantly increased their time on the mesh, indicating that they could detect the presence of conspecifics and respond to this social stimulus. Time spent with or in proximity to conspecifics is the accepted measure of sociability in vertebrate studies, and is corroborated by dyadic interactions and olfactory investigation whilst in proximity (Williams et al., 1992; Moy et al., 2004). In this study, the hive-reared honey bees that spent much of the test time on the mesh sides were also much more likely to have a two-way interaction with a stimulus bee involving antennal contact.

Having established that the new assay and measurements were compatible with honey bees, we could then investigate the development of their sociability. High levels of sociability were seen from 1 day of age in bees raised normally in their natal hive, and remained for the duration of the testing period (20 days of age). The early onset of sociability reported here also corresponds with evidence that bees acquire or produce group olfactory cues within the first 24 h post-emergence (Breed et al., 1995) and proposes an interesting parallel with data showing that rat pups express olfactory learning neonatally (Miller and Spear, 2009). By contrast, isolated bees showed a very clear reduction in their level of sociability from 1 day of age (Fig. 4) and were also far less likely to engage in dyadic interactions with another bee compared with hive-reared bees. Alterations in the frequency of both aggressive interactions and trophallaxis between ants following a period of isolation have previously been reported (Boulay and Lenoir, 2001; Seid and Junge,

Journal of Experimental Biology (2018) 221, jeb173054. doi:10.1242/jeb.173054

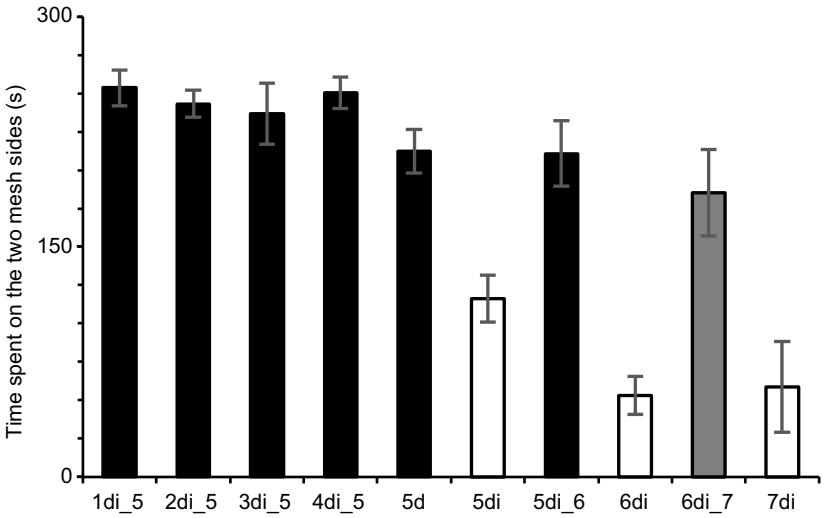

Fig. 6. No evidence of a critical period for sociability within the first 5 days post-eclosion. Bees that experienced their natal hive prior to 6 days of age (black bars) showed comparable levels of sociability to 5-day-old hive-reared bees and differed significantly from bees isolated until testing (white bars) (Kruskal–Wallis: χ^2=67.81, d.f.=9, P<0.0001, *post hoc*: a>b, $P\leq$0.01). Bees exposed to their natal colony at 6 days of age and tested the next day (grey bar) spent a comparable time on the mesh sides to all other groups (*post hoc*: ab<a, >b, P=0.13–1.00). Bars are means±s.e.m., N=199, n=9–42.

2016), but we have shown that the gregarious nature of bees, so fundamental to their survival, is also dependent on the social environment they experience post-eclosion, and not purely innate. We also showed that experience of the natal colony is crucial for high levels of sociability to persist. Sociability was further reduced in bees isolated for more than 3 days of age (Fig. 4) and, curiously, 3 days of age as an adult is also marked with an as yet unexplained spike in juvenile hormone (Kaatz et al., 1992; Jassim et al., 2000). Juvenile hormone is a prominent hormone modulating division of labour and may also be implicated in the triggering of continued sociability.

In socially deprived vertebrates, there are critical time points in development after which social contact cannot establish expression of normal social behaviours and these correlate with physiological changes during development (Scott, 1962; Bouet et al., 2011; Prounis et al., 2015). Exposing honey bees to their natal hive after varying lengths of isolation showed that the isolation-induced loss of sociability could still be recovered after the age at which bees were mature enough to have begun a range of in-hive tasks. Individual behavioural plasticity throughout a bee's lifetime is an important component of their group success as they can respond to the changing needs of the colony (Huang and Robinson, 1992). Although there is a general connection between division of labour and age (Robinson and Huang, 1998; Page and Peng, 2001), social interactions are an important driver of task allocation (Johnson, 2003; Huang and Robinson, 1992; Toth et al., 2005). Isolated bees received no social inhibition from eclosion and this may have massively accelerated them towards onset of foraging behaviour, as seen when bees forage precociously because of a shortage of older foragers in the colony (Huang and Robinson, 1992; Leoncini et al., 2004a,b). This interpretation is supported by isolation reducing proteolytic activity much earlier in young bees comparable to that of foragers (Crailsheim and Stolberg, 1989). Also, hive exposure reversed the effects of isolation in both cases, here on levels of sociability, and previously on nutritional physiology (Crailsheim and Stolberg, 1989).

Bees learn to associate a sucrose reward with gustatory and olfactory information received via a single trophallactic event (Gil and De Marco, 2005), and younger pre-foragers also associate both olfactory and tactile stimuli with a sucrose reward (Scheiner et al., 2001). It is possible that development of bee sociability could be in part a learning process involving an association of food with another bee. Isolation would prevent such learning and low levels of sociability may be a consequence. Moreover, as bees remain sensitive to social stimuli into adulthood, exposure to the hive and presumed interactions with other bees would enable this conditioned learning. Vertebrates value social stimuli as rewarding (Borland et al., 2017) and can associate social cues with a location (Calcagnetti and Schechter, 1992). Furthermore, vertebrates respond to social cues as they do to food rewards (Mühlhoff et al., 2011) and the same mesocorticolimbic system is involved (Dölen et al., 2013). However, social memories and attraction to particular conspecifics use separate neural systems (Ferguson et al., 2000). In mammals, classic studies have identified the neurobiological basis of a social encounter and its interaction with reward systems that can result in an enduring pair bond (Young and Wang, 2004). Details of the reward pathways in insects are known (Sovik et al., 2015), and many genetic and neural correlates for division of labour have been discovered. Using the assay developed here, we could uncover the mechanisms driving bee sociability and determine the developmental mechanism involved.

Although the recognition system in honey bees has been extensively investigated (Breed, 2003), a preference test using conspecifics as stimuli, using non-guard bees and focusing on an individual's response, is novel. It is important to note that although interlinked, NM recognition and affiliation are separate phenomena and this study demonstrates that. More time spent with NM than NON indicates the animal can discriminate between familiar and unfamiliar, and prefers to affiliate with NM. When no difference in time spent with each social group is recorded, we can say the animal did not express a preference to affiliate with NM over unfamiliar conspecifics but cannot conclude on their recognition abilities. Overall, hive-reared bees showed preferential affiliation to NM whereas isolated bees did not, implying that this behavioural phenomenon is also dependent on early life experiences, rather than innate preferences. This result corroborates previous work deciphering the environmental source of recognition cues used by bees and ants (Breed and Stiller, 1992; Breed et al., 1995; van Zweden and d'Ettorre, 2010). Ontogenetic effects are also evident in vertebrates as fish prefer to associate with those they grew up with rather than genetically related unfamiliar fish (Engeszer et al., 2004). Hence, as demonstrated previously and in this study, the early social environment experienced by both vertebrates and insects affects behaviour and preferences.

Previous studies on honey bee nestmate recognition implicated olfactory cues acquired from the wax comb in the hive (Breed et al., 1988, 1995, 1998); hence, bees raised away from their natal colony post-eclosion were not expected to learn and recognise their NMs.

However, as their overall sociability levels were reduced, a NM preference may simply be harder to detect. Furthermore, the NM preference reported in hive-reared bees was comparatively weak compared with their sociability scores and was not detected in any of the test groups in the second experiment, even the completely hive-reared bees. Relatedness and familiarity of the colonies used is unknown and all were located in the same bee yard and plain white hive boxes used, an arrangement that would allow drifting of bees between colonies (Pfeiffer and Crailsheim, 1998). However, the strength and variability in NM affiliation seen in this study is consistent with previous recognition studies that used guard and non-guard bees in NM recognition assays away from the context of the hive (Couvillon et al., 2013, 2015). In this assay, either friendly approach behaviour or an aggressive attack would still be recorded as time spent on their mesh. However, no aggressive behaviour was recorded, and dyadic interactions with NM and NON observed were of the same exploratory and friendly nature, supporting the possibility that dyadic interactions, regardless of bee identity, could be associated with a food reward (Gil and De Marco, 2005).

Combining the bioassay developed in this study, and knowledge gained of the development of bee sociability, with pharmacological manipulations will allow study of the physiological mechanisms underlying honey bee sociability. Having established that honey bee sociability is experience dependent, future studies can examine what specific components of the hive environment contribute to the development of honey bee sociability and when. Recognition odours acquired by bees soon after emergence and throughout adulthood have been demonstrated to come from several sources (Breed et al., 1995; Downs and Ratnieks, 1999; D'Ettorre et al., 2006). Sequential removal of each source from the environment prior to testing, and using colonies differing genetically and located apart, in the present assay will determine whether they are also important in the learning of social odour cues. Eusocial societies require individuals to balance their own and group needs (Lihoreau et al., 2014) and this assay, paired with isolation, allows comparison of bee behaviour and physiology under both solitary and social circumstances, which will help investigation of the function of bee sociability.

Acknowledgements
We would like to thank Xujiang He and Shaun Garvey for their work in the apiary and Ken Cheng and Stephan Leu for comments on earlier manuscript versions. We also thank Thomas Pyne for help with video data extraction. Marcus Plath produced the honey bee graphic in Fig. 1.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.E.H., A.B.B.; Methodology: S.E.H.; Formal analysis: S.E.H., A.B.B.; Investigation: S.E.H., D.M.W.; Data curation: S.E.H., D.M.W.; Writing – original draft: S.E.H.; Writing – review & editing: S.E.H., A.B.B.; Supervision: A.B.B.; Project administration: A.B.B.; Funding acquisition: A.B.B.

Funding
S.E.H. is funded by an International Macquarie University Research Excellence Scholarship. A.B.B. is supported by an Australian Research Council Future Fellowship number FT 140100452.

Data availability
Data are available from the Dryad Digital Repository (Hewlett et al., 2017): https://doi.org/10.5061/dryad.dj3v81b

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