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RESEARCH ARTICLE

Effects of group size on learning and memory in the honey bee *Apis mellifera*

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ABSTRACT

In animals that experience interactions with conspecifics while young, social interactions appear to be a necessary prerequisite for typical behaviour. Eusocial insects have large colonies where individuals experience a large number of social interactions with nest mates during all life stages, making them excellent candidates for understanding the effects of social isolation on brain development and behaviour. Here, we used the honey bee *Apis mellifera* to study the effect of social isolation and group size on reward perception and discrimination learning and memory. We confined day-old adult workers into three different size groups (1, 8 or 32 bees) for 6 days during a critical period associated with adult behavioural maturation. We quantified their sucrose responsiveness, their ability to use and remember olfactory cues to discriminate between sucrose and salt (i.e. discrimination learning), and four biogenic amines in the brain. We found that the smaller the group size, the more responsive a worker was to the sucrose reward. Honey bees raised in groups of 32 performed the best in the learning trials and had the highest levels of dopamine. We found no effect of group size on memory. The observed group size effect on learning but not memory supports the hypothesis that social interactions modulate learning through the dopaminergic system.

KEY WORDS: Social behaviour, Eusociality, Social isolation, Biogenic amines, Dopamine, Social interactions

INTRODUCTION

Social interactions are required for typical development in animals and social isolation often leads to atypical brain development and behaviour (Harlow et al., 1965; Fone and Porkess, 2008; Koike et al., 2009; Pan et al., 2009). In rats, for example, social isolation often leads to hyperactivity (Morgan, 1973; Syme, 1973; Einon and Morgan, 1978; Gentsch et al., 1981), increased responsiveness to amphetamines (Jones et al., 1990; Jones et al., 1992; Lapiz et al., 2003) and reduced accuracy in spatial memory (Lu et al., 2003; Quan et al., 2010; Quan et al., 2011). Similarly, insects that typically interact with conspecifics also exhibit atypical behaviour when raised in isolation. Fruit flies, which are surrounded by conspecifics during their larval stage, exhibit aggressive behaviour when raised in isolation (Valzelli and Garattini, 1972; Wang et al., 2008) and have reduced mushroom body fibres (Technau and Technau, 2007) – an insect brain region associated with learning and memory. Socially isolated carpenter ants exhibit less exploratory behaviour and have smaller mushroom bodies relative to ants raised in groups (Seid and Junge, 2016). Cockroaches, which normally live in groups, exhibit exploration avoidance, reduced willingness to interact socially, and reduced ability to assess mating partners when raised in isolation (Lihoreau et al., 2009). Additionally, isolated honey bees have reduced interactions with conspecifics and no preference in interacting with nest mates over non-nest mates, unlike hive-raised bees (Hewlett et al., 2018). As the above literature suggests, isolation in normally social organisms leads to behavioural, structural and biochemical abnormalities in both vertebrates and invertebrates.

Social insects provide outstanding opportunities for understanding how social interactions influence brain development and behaviour. Social insects live in colonies composed of many individuals and certain species, such as the honey bee *Apis mellifera*, are already model organisms for studying social behaviour and learning and memory (Menzel, 1990; Menzel, 2001; Giurfa, 2007). Honey bees are eusocial, and individual bees are surrounded by thousands of conspecifics during all life stages (Winston, 1991). Female workers interact with thousands of sisters and work cooperatively to feed the brood, maintain the hive, and forage for pollen and nectar. Honey bee workers are relatively easy to study experimentally with a plethora of behavioural testing paradigms (Menzel and Muller, 1996; Scheiner et al., 2004; Tsvetkov et al., 2018), making the species ideal for studying the influence of social interactions on ecologically relevant traits, such as learning and memory.

A few studies have examined the effects of limited social stimuli on learning and memory in *A. mellifera* workers. Ichikawa and Sasaki (2003) showed that the longer a worker is isolated following emergence, the worse her performance is in olfactory learning and memory compared with that of hive-raised bees. Importantly, they also showed that the timing of the isolation is critical – bees isolated from day 0 following emergence performed worse than those isolated from day 6. However, as the authors noted themselves, it is difficult to discern whether the deficits in learning and memory are a consequence of reduced social interactions or other typical in-hive conditions. Maleszka et al. (2009) compared olfactory learning in honey bee workers maintained in isolation with that of workers kept in groups of 50, and found that isolated workers had lower learning accuracy in a one-trial association task. While it is tempting to conclude that isolated bees suffer from learning deficits, Maleszka et al.'s (2009) findings, as well as Ichikawa and Sasaki's (2003), can indicate a deficit in learning, a deficit in reward perception, or both. Sucrose responsiveness is known to affect learning in honey bees (Scheiner et al., 2001; Scheiner et al., 2003; Scheiner, 2004), and we do not currently know whether sucrose responsiveness is influenced by social interactions. Sucrose responsiveness is also affected by the behavioural state of the bee, with foragers having higher responsiveness than nurses (Behrends et al., 2007), and the behavioural state of the bee is affected by a multitude of environmental

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factors, which include the social environment (Winston, 1991). More work is needed to understand the potential mechanisms linking social interaction and learning and memory in honey bees.

Based on mammalian work, we know that social isolation impacts learning and memory, but also responsiveness to reward and biogenic amine titers (Fone and Porkess, 2008). In honey bees, the effects of sugar responsiveness on learning and memory are well documented, with more sensitive bees having consistently higher learning scores (Scheiner et al., 2001; Scheiner et al., 2003; Scheiner, 2004). Sucrose responsiveness is impacted by biogenic amines as well. For example, injections of octopamine or tyramine increase sucrose responsiveness, while dopamine injections decrease sucrose responsiveness (Scheiner et al., 2002; Scheiner et al., 2017). In addition, biogenic amines have an impact on learning and memory that is independent of sucrose responsiveness. Aversive learning is modulated by both dopamine and serotonin in bees, via two separate pathways (Wright et al., 2010), while memory is enhanced by octopamine, but is diminished by serotonin (Erber et al., 1993). In this study, we examined how isolation and group size influence sucrose responsiveness, learning and memory, and four biogenic amines in honey bees. Specifically, we tested the following hypotheses: socially isolated honey bees will be more responsive to sucrose, based on mammalian literature (Jones et al., 1990; Harmer and Phillips, 1998; Hall et al., 1998b); socially isolated honey bees will have poorer learning performance than bees raised in groups (Ichikawa and Sasaki, 2003; Maleszka et al., 2009); and finally, the amount of dopamine and/or serotonin will be affected by social isolation, again based on the mammalian literature (Fone and Porkess, 2008).

MATERIALS AND METHODS

Bees

The bees were taken from an apiary located at the York University Research Apiary (Toronto, ON, Canada). The bees had a mixed genetic ancestry with major contributions from the East European population group (C group: *A. mellifera ligustica* and *A. mellifera carnica*) and minor contributions from the West European population group (M group: *A. mellifera mellifera*) (Harpur et al., 2012, 2013; Harpur and Zayed, 2013). We collected brood frames from two honey bee colonies maintained at the apiary in the summer of 2013. Brood frames were kept at 33°C and were checked for emerging bees daily. Every 24 h, we randomly assigned newly emerged honey bee workers to a group of 32 bees, 8 bees or 1 bee. Groups were housed in a 908 ml container (12.5×12.5×6.5 cm) with air holes in a 33°C incubator (separate from the brood); 30% sucrose (Sigma) and a pollen patty (Bee-Pro Patties, Mann Lake Ltd, Hackensack, MN, USA) were provided in excess. Dead honey bees were removed daily and if more than 20% mortality was observed in groups of 8 or 32 bees, that particular group was not used in testing. This occurred in 7/139 boxes. No more than 4 bees were tested from a single box of 8 or 32 bees. A total of 48 boxes of 8 bees and 49 boxes of 32 bees were tested.

Research on mammals indicates that a sensitive period exists at the beginning of an individual's life during which social isolation leads to atypical behaviour that is irreversible (Fone and Porkess, 2008). In adult honey bee workers, there are large shifts in neurogenomic states that occur between 1 and 6 days of age (Whitfield et al., 2006; Zayed and Robinson, 2012) and bees isolated immediately following emergence perform worse than those isolated from day 6 (Ichikawa and Sasaki, 2003). Moreover, reliable acquisition and retention of olfactory learning cannot be achieved before honey bees are at least 6 days of age (Ray and Ferneyhough, 1997). Based on this knowledge, we hypothesized that the first 6 days of a worker's adult life represent an ideal window to study how social interactions and group size influence worker behaviour.

Sucrose responsiveness

We used a standard protocol for measuring sucrose responsiveness in honey bees (Scheiner, 2004; Scheiner et al., 2004). Briefly, when the honey bees were 6 days old, they were chilled at −20°C for about 2 min, until they became immobile. They were then harnessed using a modified 1000 µl pipette tip with the tapered end removed and Plasticine was used in order to secure their thorax and legs. The bees were fed 1 µl of 30% sucrose and were left for 1.5 h on the bench top to recover, following standard methods (Frost et al., 2011). Both antennae were touched with a droplet of sucrose solution and the absence or presence of the proboscis extension was recorded. The sucrose solutions were applied in ascending order of concentration: 0%, 0.1%, 0.3%, 1.0%, 3.0%, 10% and 30%. The inter-trial interval (ITI) was 3 min. The gustatory response score (GRS), which is a measure of sucrose responsiveness (Scheiner, 2004), was calculated by summing the total number of proboscis extensions made by an individual honey bee. Thus, the possible GRSs ranged from 1 to 7, where 7 represents the most responsive bees. Honey bees that failed to respond to 30% sucrose were excluded from further testing as the reward in the discrimination learning was 30% sucrose (no significant difference was found between group size and failure to respond to 30% sucrose: chi-square test χ^2=1.73, d.f.=2, P=0.421). After testing for sucrose responsiveness, the bees were immediately tested for discrimination learning as described below.

Discrimination learning

We measured discrimination learning using a well-established (Bitterman et al., 1983) olfactory conditioning procedure by measuring the proboscis extension response with the method adapted from Ben-Shahar et al. (2000). We tested honey bees in a well-ventilated area, using either geraniol (Sigma-Aldrich, St Louis, MO, USA) or 1-hexanol (Sigma-Aldrich) as the conditioned odour stimulus (CS) and either salt (3 mol l^{-1} NaCl; Sigma-Aldrich) or 30% sucrose as the unconditioned stimulus (US). We delivered each odour as follows: 1 µl of undiluted odour solution was placed onto a filter paper located at the tip of a syringe. The bee was exposed to a 6 s puff of odour by pushing the air through the syringe. While the odour was being delivered, we placed a droplet of the US on the antennae and if the solution was sucrose, the honey bee extended her proboscis and was allowed to feed for 1 s. The learning phase consisted of 12 trials, 6 with one odour paired with sugar as a reward (CS+) and 6 with the other odour paired with salt as a punishment (CS−) in a pseudorandom order. The ITI was 3 min and proboscis extension was recorded for each trial. If the honey bee responded spontaneously to the initial odour presentation, we removed her from testing. Both odours were used as the rewarded (CS+) and punished (CS−) stimuli in different blocks, where all three group sizes were tested in a block. We tested 1 h memory by exposing the bees to CS+ and then to CS− without reward or punishment. We measured 24 h memory by exposing the bees to CS− and then to CS+. A correct response was classified as extending the proboscis in response to CS+ but not to CS−. After the 24 h memory test, the bees were immediately placed in dry ice and then stored at −80°C.

Brain dissections

We analysed four biogenic amines of bees from all three groups with GRS scores of 1, 4 and 7. First, the honey bee heads were freeze dried at −80°C, 42 Pa for 60 min (Schulz and Robinson, 1999;

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Labconco Triad System, Kansas City, MO, USA; with Welch 8917 vacuum pump, Mt Prospect, IL, USA). Then, the exoskeleton was removed and the size of the hypopharyngeal gland was scored as described below. Afterwards, it was removed, allowing for a clean excursion of the brain. Three individual brains were grouped based on group condition and GRS score into one 1.5 ml Eppendorf tube and stored at −80°C until biogenic amine analysis (N=35) (Hartfelder et al., 2013).

A potential confounding factor in our analysis is a change in the behavioural roles of the bees. Although, typically, young bees are nurses and older bees are foragers, worker honey bees have a flexible division of roles based on the surrounding environmental conditions (Winston, 1991). It is possible that socially isolated bees may be forager like, while the non-isolated bees may be nurse like. In order to explore this possibility, we measured the size of the hypopharyngeal glands while dissecting the brains by classifying them into six arbitrary categories from 1 (totally undeveloped) to 6 (fully developed) (Free, 1961). Foragers have less developed hypopharyngeal glands than nurses, regardless of age (Huang and Robinson, 1996). This should thus reveal any changes in behavioural roles and whether they correspond to the differences in learning and memory that we might detect.

Biogenic amines

We used high performance liquid chromatography (HPLC) to quantify octopamine, dopamine, tyramine and serotonin. To extract amines from brain tissue, we removed Eppendorf tubes containing brains from the −80°C freezer and placed them on ice. We added 20 µl of 0.2 mol l^{-1} perchloric acid with internal controls dihydroxybenzylamine (DHBA, 87 pg $µl^{-1}$; Sigma-Aldrich) and synephrine (50 pg $µl^{-1}$; Sigma-Aldrich) to each tube. We used a plastic pestle to disrupt brain tissue for 30 s. We then placed tubes containing brains into a sonicated ice bath for 5 min, after which they were allowed to incubate for 20 min to further extract amines. We then centrifuged the brains at 12,000 ***g***, 4°C for 10 min to pellet the tissue (Wagener-Hulme et al., 1999; Hartfelder et al., 2013; Cook et al., 2017).

We used a 10 µl glass syringe (Hamilton) to manually inject 10 µl of the amine-containing supernatant into the HPLC system (Coularray 5600A, ESA, Chelmsford, MA, USA) with a reverse-phase catecholamine HR-80 column (Thermo Fisher Scientific, Waltham, MA, USA). The sample was initially held in a 20 µl holding loop, then manually injected into the system (Rheodyne 9125, Rohnert Park, CA, USA). The sample then passed through a 4-channel electrochemical detector, with channel voltages set at −125, 175, 425 and 640 mV. The detection limit for the HPLC is 0.2 ng at the 640 mV channel, which was used for quantification of all amines. The mobile phase consisted of 15% methanol, 15% acetonitrile, 85 mmol l^{-1} sodium phosphate monobasic, 5 mmol l^{-1} sodium citrate, 1.5 mmol l^{-1} sodium dodecyl sulfate and polished water. The pH was adjusted to 5.6 using phosphoric acid. The mobile phase flow rate was 1 ml min^{-1}. All results are reported in picograms per three brains and were calculated from curves of external standards run before, during and after samples (Penick et al., 2014; Brent et al., 2016; Cook et al., 2017) (hydrochloric forms of dopamine, octopamine, serotonin and tyramine; Sigma-Aldrich).

Statistics

Statistical analyses were performed using R (version 3.1.1) (R Core Team, 2005; http://www.R-project.org/). The discrimination learning and memory data were analysed using a generalized linear model, with binomial family distribution for the latter. All model residuals were inspected. For the biogenic amine data, we first normalized the data, removed outliers using Cook's D (Cook, 1977), and then carried out a multivariate regression. A total of five data points were removed as outliers.

RESULTS

Increased group size was associated with decreased sucrose responsiveness. All group conditions were significantly different from the other conditions (Fig. 1; pairwise Wilcox test, Holm–Bonferroni P correction, group 1 versus 8: P=0.003; group 1 versus 32: P<0.001, group 8 versus 32: P=0.008).

As expected from previous studies (Scheiner et al., 2004), the bees with the highest GRS had the most correct responses during the learning trials (Fig. 2; GLM, t=5.403, P<0.001) and were more likely to respond correctly during the 1 h memory tests (GLM, z=2.759, P=0.005). A marginally significant relationship was found between GRS scores and 24 h memory (GLM, z=1.828, P=0.068).

Bees raised in groups of 32 had the most correct responses during the learning trial (Fig. 2; GLM, t=2.554, P=0.011). Group size did not have an effect on 1 h (GLM, z=0.542, P=0.588) or 24 h (GLM, z=0.517, P=0.605) memory.

Honey bees raised in isolation had a significantly smaller hypopharyngeal gland than those raised in groups of 8 or 32 (Fig. 3; GLM, t=2.707, P=0.008), but we found no relationship between hypopharyngeal gland size and GRS score (GLM, t=–0.037, P=0.971) nor any learning and memory performance (GLM, P>0.05).

The overall levels of all four biogenic amines are comparable to previous studies (Table S1; Wagener-Hulme et al., 1999; Scheiner et al., 2017). A multivariate regression analysis on the brain biogenic amine data revealed an effect of group size on dopamine levels [Table 1; linear regression model (lm), t=2.756, P=0.011]. Bees raised in groups of 32 had the highest mean dopamine levels with 879 pg per sample of three brains, while bees raised in groups of 8 had a mean of 725 pg per sample and for bees raised in isolation the mean was 788 pg per sample. Group size did not have a statistically significant effect on serotonin, octopamine or tyramine levels (Table 1, P>0.05). We found a negative relationship between gland development and tyramine (lm, t=−2.513, P=0.019) as well

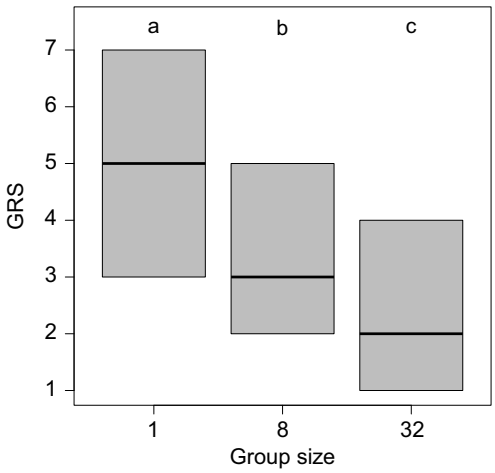

Fig. 1. Sucrose responsiveness of honey bees raised in different social conditions. The more bees a worker was raised with, the lower her gustatory response score (GRS) (pairwise Wilcox test P<0.01; n_1=83, n_8=86, n_{32}=97). The median (bold line) and quartiles (upper and lower lines) are displayed.

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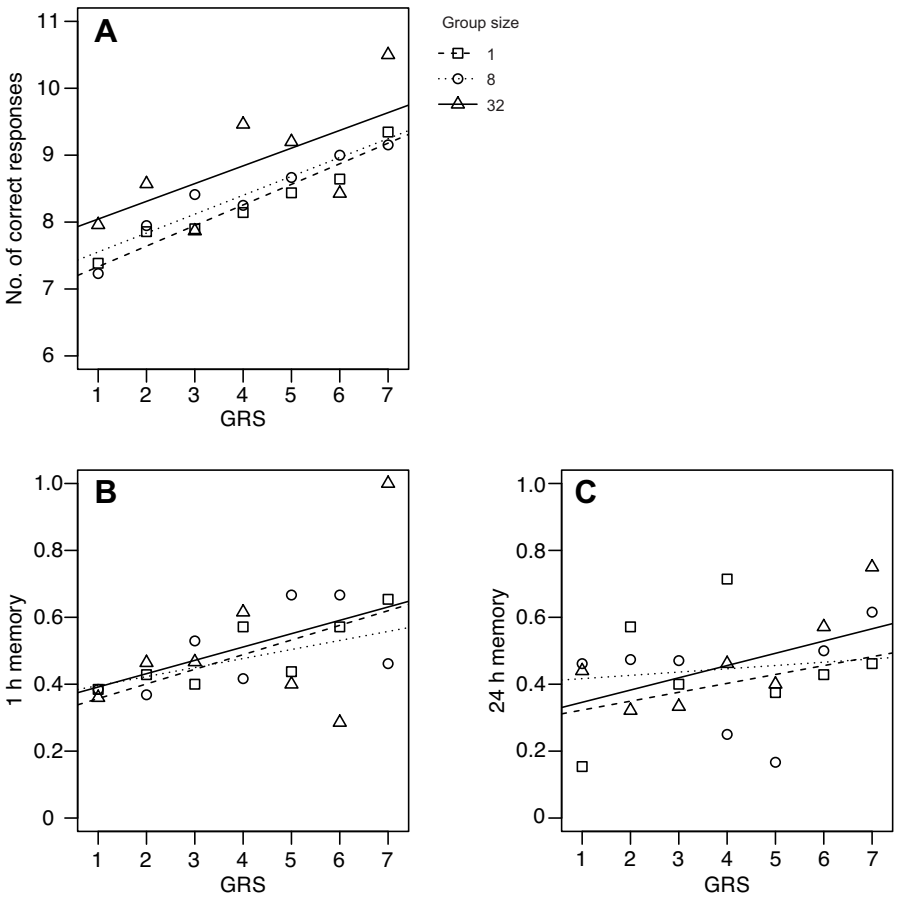

Fig. 2. Discrimination learning of honey bees raised in different social conditions. Bees raised in groups of 32 had the most correct responses during the learning trials (A, GLM, P=0.011; n_1=83, n_8=86, n_{32}=97), but group size had no effect on 1 h (B, GLM, P=0.588) or 24 h (C, GLM, P>0.605) memory. Bees with higher sucrose responsiveness (GRS) had more correct responses during the learning trials (A, GLM, P<0.001) and higher performance in the 1 h memory test (B, GLM, P=0.005), but not in the 24 h memory test (C, GLM, P=0.068). The mean (symbols) and the line of best fit are displayed.

as octopamine (lm, t=−2.162, P=0.040) levels. No other statistically significant effect of group size, gland development or GRS was found on the four tested biogenic amines (P>0.05).

DISCUSSION

In order to investigate the effects of social isolation and group size on learning and memory in honey bees, we randomly assigned emerging honey bees to one of three different group sizes (1, 8 or 32 bees). We found that group size affected sucrose responsiveness, where bees raised in smaller groups were more responsive to sucrose. These results are in line with literature on other animals, where rats raised in isolation are more sensitive to low saccharine solutions (Hall et al., 1998b), consume more sucrose solution (Hall et al., 1997; Hall et al., 1998b) and are more responsive to food rewards (Jones et al., 1990; Harmer and Phillips, 1998). We find the large effect of group size on sucrose responsiveness to be particularly intriguing; the reward system is heavily intertwined with behaviour, and there is a burgeoning body of literature suggesting that social evolution involves changes in the regulation of the reward system (Søvik et al., 2015). Our finding that group size influences sucrose responsiveness is consistent with ideas regarding social control of the honey bee reward system.

Our experiment shows that reduced social interactions induce learning deficits that are not mediated by reduced reward perception. These results are consistent with those of Maleszka et al. (2009) and Ichikawa and Sasaki (2003), who showed that honey bees raised in isolation have worse learning and memory performance than those raised in groups of 50 or in the hive, respectively. No effect of group size was detected on 1 h or 24 h memory. Learning and memory are mediated by different molecular processes (Menzel and Muller,

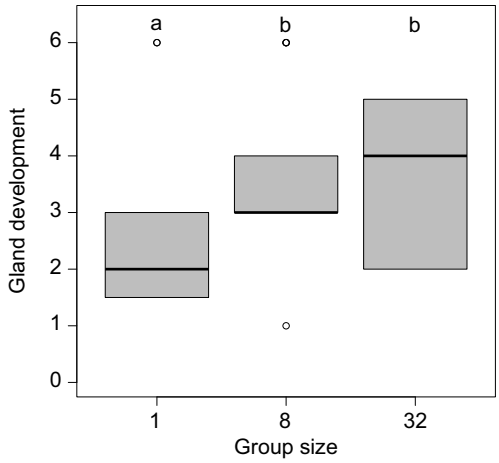

Fig. 3. Gland development of honey bees raised in different social conditions. Bees raised in isolation had the smallest hypopharyngeal gland development scores (GLM, t=2.707, P=0.008; n_1=36, n_8=36, n_{32}=32). The median (bold line) and quartiles (upper and lower lines) are displayed.

Table 1. Odds-ratio of the multivariate regression analysis of biogenic amines

	Serotonin	Octopamine	Dopamine	Tyramine
Group size	1.017	1.043	**0.994**	1.017
Gland development	0.677	**0.684**	0.759	**0.618**
GRS	1.093	1.024	1.136	1.017

Bold values are statistically significant (P<0.05).

1996), so it is possible that group size in honey bees influences the processes implicated in learning, but not memory.

We found that bees in isolation had the smallest hypopharyngeal glands, which is in agreement with previous work (Suzuki, 1988), and we found a negative relationship between gland development and both tyramine and octopamine levels. As tyramine is a precursor for octopamine, but can also act independently (Roeder, 2005), it is difficult to discern its role in this case. Octopamine, among other things, regulates worker division of labour in honey bees (Schulz et al., 2002), where treatment with octopamine initiates foraging behaviour (Schulz and Robinson, 2001). Foragers also have smaller hypopharyngeal glands than nurses (Huang and Robinson, 1996). This might suggest that our socially isolated bees were more forager like. Behavioural roles in honey bees are malleable and responsive to the environment (Winston, 1991). Foragers have a higher sucrose responsiveness than nurses (Behrends et al., 2007) and our isolated bees had the highest sucrose responsiveness. However, nurses and foragers exhibit no significant differences in discrimination learning (Ben-Shahar and Robinson, 2001), which does not agree with our results. In addition, foragers have higher levels of tyramine (Scheiner et al., 2017), octopamine and dopamine (Wagener-Hulme et al., 1999), but we only found a significant difference in dopamine levels and they were highest in our largest social group, which, hypothetically, would have been the most nurse like. As such, the relationship between group size and learning cannot be fully explained by differences in behavioural roles of the isolated bees relative to bees raised in groups. In addition, all of the bees were raised without the presence of queen pheromone. Naeger et al. (2013) showed that the absence of a queen results in more blurring between the behavioural roles of workers: bees that were previously foragers start performing typical nurse duties and vice versa.

Dopamine was the only biogenic amine that was influenced by group size. Mean dopamine levels were highest in bees raised in groups of 32. It is possible that the dopaminergic system is disrupted by the reduced group size in honey bees. In rats, the dopaminergic system has been implicated in food responsiveness and consumption (Sills and Crawley, 1996; Hajnal et al., 2004; Avena et al., 2008) and it is also disrupted during isolation (Blanc et al., 1980; Jones et al., 1990, 1992; Hall et al., 1998a; Fabricius et al., 2010; Yorgason et al., 2013). In honey bees, injections of dopamine and the dopamine receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN) reduce sucrose responsiveness (Scheiner et al., 2002), and dopamine injections before training do not alter memory retrieval or storage (Mercer and Menzel, 1982; Menzel et al., 1988, 1990). The dopaminergic system is involved in aversive conditioning in honey bees and other insects (Giurfa, 2007), where dopamine antagonists impair aversive acquisition (Unoki et al., 2005; Agarwal et al., 2011). As discrimination learning involves aversive acquisition (odour with salt punishment), it is probably affected by dopamine. Thus, lower levels of dopamine in the smaller groups would be expected to cause higher sucrose responsiveness and impaired learning performance, but not affect memory, which is consistent with the results of this study.

We failed to detect significant differences in octopamine, serotonin and tyramine levels between the different social groups. This result should be interpreted with caution, as it might be due to the low sensitivity of our methods. Although Schulz and Robinson (1999) found no difference between freeze-dried and snap-frozen bees, snap freezing is an ideal method as it is more rapid and widely used in behavioural work (Chen et al., 2008; Sasaki and Harano, 2007; Cook et al., 2017). Additionally, analysing individual honey bee brains would facilitate a more robust comparison and identification of individual variation (Cook et al., 2019). Further, we used an HPLC with four channels to measure and identify biogenic amines. Using single- or double-channel identification could reduce the time the amines spend in the system, reducing degradation (Muscedere et al., 2012; Wagener-Hulme et al., 1999). Reducing the time spent processing the biogenic amines and measuring individual brains in the future could allow us to identify more minute changes associated with different social environments. However, we note that all samples were processed in the same manner and thus the significant associations between some amines and the conditions tested in Table 1 are unlikely to be spurious artefacts of sample handling.

The variety of social conditions seen in insects provides a good opportunity to study social isolation and group size effects on behaviour. Studying insects that are solitary, communal or social, and manipulating their social conditions at particular life stages would illuminate how crucial social interactions are for typical behaviours. Our study focused on a eusocial insect and demonstrated that group size during a certain developmental stage is important for learning. Other studies have focused on insects that are less social (Technau and Technau, 2007; Wang et al., 2008; Lihoreau et al., 2009), but have also found that the social environment has an effect on behaviour. Using insects with different social structures would allow us to make more direct comparisons on the effects of social interactions in general. The diversity of social lifestyles within insects makes them a good model for studying the effects of social interactions on behaviour.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.T., A.Z.; Methodology: N.T., C.N.C.; Formal analysis: N.T.; Investigation: N.T., C.N.C.; Resources: A.Z.; Writing - original draft: N.T., A.Z.; Writing - review & editing: N.T., C.N.C., A.Z.; Visualization: N.T.; Supervision: A.Z.; Project administration: N.T., A.Z.; Funding acquisition: A.Z.

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