

SHORT COMMUNICATION

Stress decreases pollen foraging performance in honeybees

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ABSTRACT

Foraging in honeybees is energetically demanding. Here, we examined whether stressors, which generally increase metabolic demands, can impair foraging performance. A controlled non-pathogenic stressor (immune challenge) resulted in a change in the foraging preferences of bees. It reduced pollen foraging and increased the duration of trips in pollen foragers. Stress also reduced the amount of octopamine in the brain of pollen foragers (a biogenic amine involved in the regulation of foraging and flight behaviour in insects). According to the literature, flight metabolic rate is higher during pollen foraging than during nectar foraging, and nectar gives a higher energetic return relative to the foraging effort when compared with pollen. We thus propose that stress might be particularly detrimental to the performance of pollen foragers, and stressed bees prefer the energy-rich resource of nectar. In conclusion, stress, even at low levels, could have consequences for bee foraging behaviour and thereby the nutritional balance of the colony.

KEY WORDS: Immune challenge, Flight, Biogenic amine, Radio-frequency identification device

INTRODUCTION

For honeybees, which are central-place foragers relying on pollen and nectar from flowers, foraging behaviour places demands on both cognitive capacity (Klein et al., 2017) and metabolic capacity: indeed, insect flight is known to be among the most intense and energy-demanding physiological processes in the animal kingdom (Dudley, 2000). The metabolic rates of flying insects, mainly fuelled by carbohydrates, can be up to 170 times higher than those of resting individuals (Bartholomew and Casey, 1978). As a consequence, it is expected that environmental stressors (e.g. parasites and temperature changes), which often impose increased metabolic demands (Bordier et al., 2017a; Johnson and White, 2009), could compromise foraging performance. Deciphering how stress impacts honeybee foraging performance might therefore help us better understand the mechanisms underlying colony decline and failure, which continues to be an issue of widespread concern (Goulson et al., 2015; Potts et al., 2010).

Stressors may directly limit bees' energetic reserves and thus reduce foraging performance. Indeed, there are several reports of a reduction of global flight activity in parasitized bees due to energy

depletion (Kralj and Fuchs, 2010; Alaux et al., 2014; Naug, 2014; Wolf et al., 2014). Stressors may also affect forager decision-making processes as a consequence of the energetic challenges of the stressor, in which case bees may show a preference for carbohydrate-rich resources to supply their own energy needs. The finding that the gene coding for the pheromone biosynthesis-activating neuropeptide, a neuropeptide known to be present at higher levels in nectar foragers than in pollen foragers (Brockmann et al., 2009), is over-expressed in parasitized bees (McDonnell et al., 2013) provides some indirect support for this hypothesis. Stress can decrease sucrose responsiveness (Pankiw and Page, 2003), which is lower in nectar foragers than in pollen foragers (Pankiw and Page, 2000), suggesting that stress might cause a change in foraging preference. In addition, it has been shown that parasitized bees are less likely to forage for pollen (Lach et al., 2015). Together, these findings suggest that stressed bees may favour nectar over pollen foraging. This could have consequences for the nutritional balance and development of the colony, as the majority of larva protein intake indirectly comes from pollen supply (Brodtschneider and Crailsheim, 2010; Pernal and Currie, 2000). Moreover, pollen nutrition promotes immunocompetence and parasitism tolerance of adult bees (Alaux et al., 2010; Di Pasquale et al., 2013).

To test the hypothesis that stress can induce a change in foraging performance, without any potential effects of parasite manipulation of host metabolism (Adamo, 2012; Biron and Loxdale, 2013), we exposed bees to a non-pathogenic immune challenge. Immune responses are energetically costly, and even simple responses, like encapsulation, can raise metabolic rate by up to 28% in insects (Ardia et al., 2012; Freitak et al., 2003). We then tracked their foraging behaviour throughout their life with a radio-frequency identification device (RFID), and a camera at the colony entrance to identify whether they carried pollen loads. Finally, we assessed the influence of stress on brain biogenic amine levels, which are known to be involved in the regulation of bee behaviour (Schulz and Robinson, 2001; Schulz et al., 2002).

MATERIALS AND METHODS

Experiments were performed from January to April 2016 with honeybees (*Apis mellifera* Linnaeus 1758) obtained from the research apiary at Macquarie University (Sydney, NSW, Australia). We tested the influence of stress on foraging behaviour (experiment 1) and brain biogenic amine signalling (experiment 2). Frames containing late-stage pupae were collected from three donor colonies and placed into an incubator overnight at 34°C. Newly emerged bees were marked on the thorax with either a RFID tag for experiment 1 or a paint mark for the experiment 2, and released into host colonies. They were then re-captured when 7 days old and placed in plastic cages with *ad libitum* sugar solution (50% w/v). Half of the bees were given an immune challenge, which consisted of piercing the cuticle between the third and the fourth tergites of the abdomen using a 0.15 mm needle. If a haemolymph drop was released after the pin prick, the bee was discarded. Previous studies have shown that the bee's immune system is activated by this

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wounding alone, without pathogen infection (Alaux et al., 2014; Evans et al., 2006; Siede et al., 2012). Control bees did not receive any pin prick. Handled bees (control and immune challenged) were given an additional paint mark on the abdomen to identify them by their treatment group before they were released back into their colony. This procedure was repeated three times with different bees.

Experiment 1: impact of immune challenge on foraging performance

Following the stress treatment at 7 days, 380 control and 370 stressed bees in total ($n=3$ trials) were released into a small nucleus hive equipped with a modified entrance. Bees had to use a specific path to exit the hive and another one to enter the hive. Each path was made of transparent 1 cm diameter plastic tubing (Bunnings, Gordon, NSW, Australia). To avoid bees using the wrong path, a plastic gate with plastic bristles, which bees could use in only one direction, was placed at the end of each path. The traffic of bees was also regulated using infrared-activated gates placed at the beginning of each path (Arduino Technology, Arduino, Adafruit and Little Bird Electronics, Hornsby, NSW, Australia). Each time a bee broke the infrared beam, the linked gates were closed behind the bee for 10 s, which was the time needed for bees to cross the path and RFID system. Each path was equipped with a RFID reader (Invengo, Guangzhou, China; Perry et al., 2015; Søvik et al., 2015) to monitor each of the entrance and exit channels. Each RFID tag (diameter 4 mm, mass 1 mg) had a unique digital identifier read by the antennae at the entrance and exit. The entrance path was also equipped with a digital video camera (Logitech, Lausanne, Switzerland) and a white LED light enclosed in a plastic box. Motion detection video recording software (ZoneTriger, Omega Unfold Inc., Montreal, QC, Canada) was used to visually identify whether bees carried pollen or not.

Experiments continued until the last recording of the last bee, i.e. 55 days. RFID data, i.e. bee ID, direction (entry into or exit from the hive) and time (day, hours, minutes and seconds), were recorded in .csv files. From these data, we were able to reconstruct trips outside the hive for each bee. RFID readings were time matched with readings from the camera, and videos taken from 10 s before RFID detection were inspected to identify the resource for the returning bees (pollen or not pollen). Only data for bees with an RFID tag and paint marks on their abdomen were analysed. Trips shorter than 30 s were not considered as foraging flights and were excluded from the study. As in Perry et al. (2015), trips longer than 8 h were also removed.

Of the 380 control and 370 immune-challenged bees, a completed foraging flight was recorded at least once from 96 and 74 bees, respectively. This loss of bees could be due to the loss of tag prior to leaving the hive, ejection from the colony by nestmates or death of the bee during its first flight. In total, 979 flights identified as pollen ($n=154$) or non-pollen (which can be nectar, water or an empty crop; $n=825$) foraging flights were recorded. The number of foraging flights appeared to be relatively low for a total of 170 bees, but was probably explained by the fact that the experimental device contained only one entry and one exit path (one bee at a time could use the path), and that many bees completed a very low number of flights (median, first and third quartiles: 4, 2, 8 foraging trips per bee, respectively). A maximum of 83 completed foraging trips per bee was recorded and 20 bees completed more than 20 trips.

Experiment 2: impact of immune challenge on brain biogenic amine levels

After the stress treatment on day 7, 637 control and 695 immune-challenged bees in total were introduced into a normal Langstroth

colony ($n=3$ trials). Bees returning to the colony when they were between 24 and 28 days old were sampled and immediately flash-frozen in liquid nitrogen. Whether they carried pollen or not was also noted. Frozen heads were freeze-dried for 60 min at a pressure below 300 mTorr (~ 40 Pa; VirTis Benchtop™) and -35°C and then stored at -80°C until brain dissection and biogenic amine analysis. Brain dissections (including optic lobes, antennal lobes, the central brain and gnathal ganglion) were performed on dry ice.

Brain biogenic amine (octopamine, OA; dopamine, DA; tyramine, TYR; and serotonin, 5-HT) levels were measured using high-pressure liquid chromatography (HPLC) following the protocol described by Søvik et al. (2013) and also used later (Scheiner et al., 2014; Søvik et al., 2015). Briefly, the HPLC system was composed of a pump and an autosampler (Agilent 1200 Series, Agilent Technologies, Santa Clara, CA, USA), coupled to an electrochemical detector (ESA Coulochem III, Chelmsford, MA, USA) connected to an analytical cell (ESA 3011A). A 100 mm Hypersil BDS octadecylsilane column was used to separate samples (ThermoFisher Scientific, Waltham, MA, USA). Signals were integrated using ChemStation software (Agilent Technologies) with reference to a standard curve obtained from perchloric acid solutions containing $10\text{ pg }\mu\text{l}^{-1}$ of dihydroxybenzylamine and varying amounts of OA, DA, TYR and 5-HT (Sigma-Aldrich).

In total, we obtained information on brain levels of biogenic amines for 94 control bees (32 with pollen and 62 without pollen) and 50 immune-challenged bees (12 with pollen and 38 without pollen). TYR was detected in only 14% of brains, and thus was not analysed.

Statistical analysis

All statistics were performed using the statistical software R version 3.2.1 (<http://www.R-project.org/>). For experiment 1, the last day any individual bee was detected using RFID was assumed to mark the date of bee death. We then compared the probability of survival between stressed and control bees using the Kaplan–Meier test ('surfit' function of the survival package in R) (Therneau and Lumley, 2014).

Aspects of the foraging performance of bees were analysed using mixed models. The choice of best-fit model was based on the smaller sample size-corrected Akaike's information criterion (AICc) (Burnham and Anderson, 2004). Variation in total number of completed foraging flights per bee, the collected resource (pollen or not pollen) and foraging trip duration were each analysed using different mixed models and fitted with a Poisson, binomial and Gaussian distribution, respectively (based on the distributions of our experimental data). To analyse the number of trips and the collected resource, the treatment (immune challenged or control) and trial were set as fixed and random explanatory variables, respectively. To analyse foraging trip duration, collected resource and honeybee identification were added as fixed and random explanatory variables, respectively.

The normality and the homoscedasticity of brain biogenic amine levels were such that parametric analyses were appropriate for these data. Biogenic amine amounts were analysed using a repeated measures ANOVA followed by Tukey's *post hoc* comparison. Treatment and the resource collected (pollen or not pollen) were analysed as fixed factors, while trial was analysed as a random factor.

RESULTS AND DISCUSSION

Experiment 1: impact of immune challenge on survival and foraging performance

Survival probability did not differ between the control and immune-challenged groups (Kaplan–Meier test, $P=0.42$; Fig. 1A).

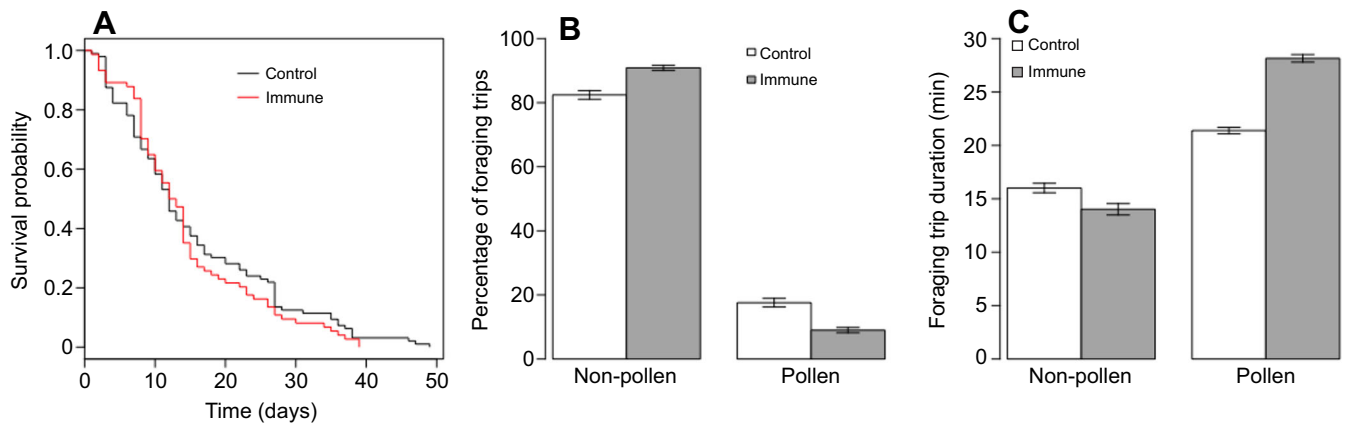


Fig. 1. Survival probability and foraging trip characteristics according to treatment. (A) Survival over 49 days for control bees and immune-challenged bees. Day 0 was the day of stress exposure. Bees from the two treatment groups did not differ in survival probability (Kaplan–Meier test, $P=0.42$). (B) Percentage of pollen and non-pollen foraging trips. (C) Foraging trip duration. For B and C, the mean and 95% confidence intervals predicted by the model (Table 1) are shown according to the collected resource and the treatment: control ($n=100$ pollen and 401 non-pollen foraging trips) and immune challenge ($n=54$ pollen and 424 non-pollen foraging trips). Immune-challenged bees performed fewer but longer pollen foraging trips than control bees.

The best-fit model explaining the variability in the number of trips per bee (lowest AICc) included a significant effect of treatment (Table 1). Immune-challenged bees completed slightly more flights than control bees [mean predicted values with 95% confidence interval: 6.46 (6.12–6.80) versus 5.22 (4.95–5.49), respectively].

A significant switch in foraging preference was detected, with immune-challenged bees performing 1.9 times fewer pollen foraging trips [9.14% (8.32%–9.96%)] than control bees [17.56% (16.20%–18.91%); Fig. 1B and Table 1].

Considering foraging trip duration, the best-fit model included a significant interaction between treatment (immune challenged or control) and the collected resource (pollen or not pollen) (Table 1). Pollen foraging trips were longer than non-pollen foraging trips (Fig. 1C), but trip duration for each collected resource also varied with treatment. Immune-challenged bees performed slightly shorter non-pollen foraging trips than control bees (Fig. 1C), but when foraging for pollen, immune-challenged bees performed 30% longer trips than control bees (Table 1).

Experiment 2: impact of immune challenge on brain biogenic amine levels

Brain DA and 5-HT levels did not differ significantly between treatment groups (ANOVA: $P=0.67$ and $P=0.14$, respectively) or the collected resource (ANOVA: $P=0.75$ and $P=0.27$, respectively; Fig. 2A,B). However, we found a significant treatment \times resource

interaction for brain OA levels (ANOVA: $P=0.02$; Fig. 2C). No difference in brain OA levels was found in non-pollen foraging bees (Tukey's *post hoc* tests: $P=1$); however, when sampled on return to the hive carrying pollen, immune-challenged bees had significantly less OA in the brain than control bees ($\sim 27\%$ less, Tukey's *post hoc* tests: $P=0.032$; Fig. 2C).

Experimental findings

In this study, we have provided experimental evidence for a stress-induced decrease in pollen-foraging performance in honeybees. The non-pathogenic immune challenge stress applied did not affect bee survival, as has been found previously (Alaux et al., 2014), but did induce a shift in resource collection. An increase in non-pollen foragers (water foragers, nectar foragers and/or bees with empty crops) was observed at the expense of pollen foragers. As more than 90% of non-pollen foragers are nectar foragers and bees with empty crops (Bordier et al., 2017b) and these bees have lower sucrose responsiveness than pollen foragers (Pankiw and Page, 2003), we could reasonably assume that stress decreased bee sucrose responsiveness. Stressed bees may prefer to forage for resources that are rich in carbohydrates to overcome the energetic cost of the stress, as has been observed with parasitism of honeybees (Lach et al., 2015). Indeed, compared with pollen, nectar gives a higher energetic return relative to the foraging effort (8:1 gain with pollen versus 10:1 gain with nectar; Winston, 1987). Similarly,

Table 1. Summary of best-fit mixed models to analyse the impact of immune challenge on foraging behaviour

Dependent variable	Explanatory variables		No. of statistical units	d.f.	AICc		
	Fixed	Random					
No. of foraging trips	Treatment	Trial	170 bees from 3 trials	3	1594.7		
	Null					2	1606.1
Foraging trip duration	Treatment \times resource	Trial/bee	979 observations of 170 bees from 3 trials	6	9120.3		
	Treatment+resource					5	9129.0
	Treatment					4	9148.3
	Resource					4	9130.9
	Null					3	9150.2
Foraging preference	Treatment	Trial	170 bees from 3 trials	3	379.2		
	Null					2	391.3

Three models were fitted to analyse the number of foraging trips, foraging trip duration and foraging preference (pollen or not pollen). Only summaries of the best-fit models are shown. For each model, fixed and random explanatory variables, the number of statistical units, degrees of freedom (d.f.) and corrected Akaike's information criterion (AICc) are detailed. For each dependent variable, the selected model, i.e. the one with the lowest AICc, is indicated in bold.

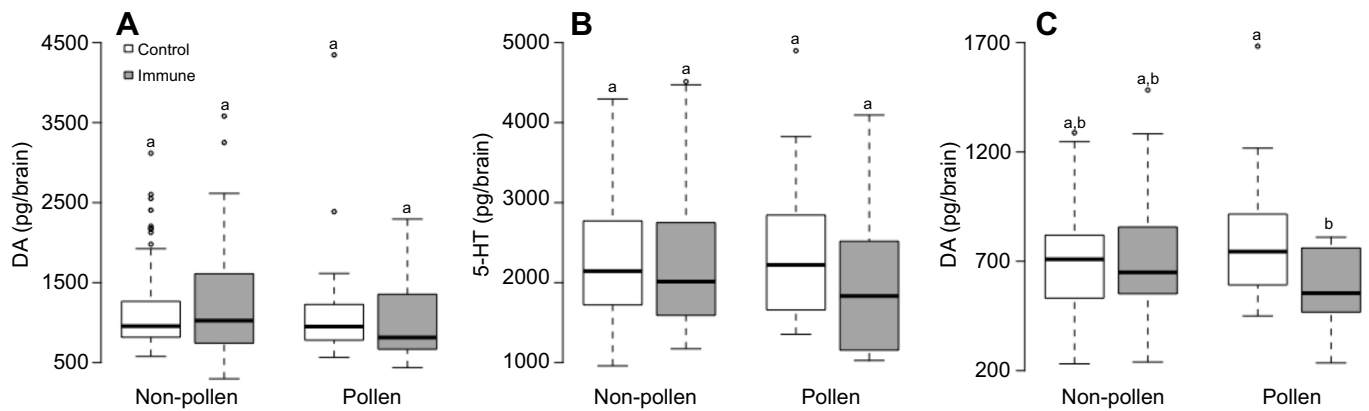


Fig. 2. Brain biogenic amine levels in response to immune challenge in different forager groups. Dopamine (DA, A), serotonin (5-HT, B) and octopamine (OA, C) levels are shown for control ($n=32$ pollen and 62 non-pollen foragers) and immune-challenged bees ($n=12$ pollen and 38 non-pollen foragers), according to the collected resource. Boxes show the first and third interquartile range with the line denoting the median. Whiskers encompass 90% of the individuals, beyond which each outlier is represented by a circle. Plots that do not share a common letter are statistically different ($P<0.05$, ANOVA followed by Tukey's *post hoc* comparisons).

bumblebees exposed to pesticides were found to exhibit lower pollen foraging performance (Feltham et al., 2014; Gill and Raine, 2014).

Such changes in foraging decision making could cause a nutritional imbalance with a pollen deficit at the colony level, and thereby affect colony development. Indeed, pollen shortage may have detrimental effects on brood care, resulting in undernourished larvae (Blaschon et al., 1999) and emerging adults with behavioural deficiencies (Scofield and Mattila, 2015). Moreover, pollen nutrition during the adult stage is essential for stress tolerance (DeGrandi-Hoffman et al., 2010; Di Pasquale et al., 2013; Wahl and Ulm, 1983). Finally, under extreme pollen shortage, nurse bees may reduce the number of larvae that need to be fed, and cannibalize eggs and young larvae (Schmickl and Crailsheim, 2001).

Pollen foraging trips were also 30% longer for immune-challenged bees, suggesting a significant effect of the stressor on foraging capacity. It has been found that the thorax temperature differs between different classes of foragers, in the order pollen>nectar>water foragers (Feuerbacher et al., 2003). These differences were linked to flight metabolic rate, with pollen foragers exhibiting a 10% higher hovering metabolic rate than nectar foragers, regardless of their loads (Feuerbacher et al., 2003). The authors suggested that pollen foragers require more power output to generate the same vertical lift as nectar foragers. We therefore propose that immune-challenged bees spend more time on pollen-collecting trips because it is the most energetically demanding resource to collect (Feuerbacher et al., 2003) and the stressor probably decreases the energy budget of the bees. The increase in foraging trip duration may simply reflect more time resting rather than any other changes in flight characteristics (e.g. distance, speed, etc.) (Wolf et al., 2014). It is also possible that a lower energy budget induced by the stressor caused cognitive impairment in pollen foragers and thus affected their navigation capacities (Jaumann et al., 2013), lengthening their trip times.

Finally, we found that brain OA level was depressed in immune-challenged pollen foragers. OA is known to increase sucrose responsiveness in bees (Scheiner et al., 2002) and stimulate flight activity (Fussnecker et al., 2006), and therefore the drop in OA level is in accordance with the behavioural changes observed in pollen foragers after stress exposure. A previous study reported a rapid decrease in OA and DA but not 5-HT levels in response to stress exposure (chilling anaesthesia and vertical spin; Chen et al., 2008).

We did not find variation in DA levels after our stress exposure. However, to conclude on the nature of the causal role of biogenic amines in honeybee stress responses, functional studies involving manipulation of OA, DA and 5-HT signalling would be required.

Conclusion

Our study suggests that the highly energy-demanding foraging activity of pollen foragers makes them susceptible to stress, even at low levels, which could potentially affect the colony nutrient balance (pollen versus nectar). Therefore, future studies on whether stress narrows the colony foraging flexibility in response to environmental changes might help us to better understand colony decline.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.B., A.B.B., C.A.; Methodology: C.B., S.K.; Formal analysis: C.B., S.K.; Resources: A.B.B.; Writing - original draft: C.B., C.A.; Writing - review & editing: C.B., S.K., Y.L.C., A.B.B., C.A.; Supervision: A.B., C.A.

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