© 2016. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2016) 219, 3003-3008 doi:10.1242/jeb.143511

RESEARCH ARTICLE

Insulin effects on honeybee appetitive behaviour

Carolina Mengoni Goñalons[1,‡], Marie Guiraud[2,*,‡], María Gabriela de Brito Sanchez[2] and Walter M. Farina[1,§]

ABSTRACT

Worker honeybees (*Apis mellifera*) carry out multiple tasks throughout their adult lifespan. It has been suggested that the insulin/insulin-like signalling pathway participates in regulating behavioural maturation in eusocial insects. Insulin signalling increases as the honeybee worker transitions from nurse to food processor to forager. As behavioural shifts require differential usage of sensory modalities, our aim was to assess insulin effects on olfactory and gustatory responsiveness as well as on olfactory learning in preforaging honeybee workers of different ages. Adults were reared in the laboratory or in the hive. Immediately after being injected with insulin or vehicle (control), and focusing on the proboscis extension response, bees were tested for their spontaneous response to odours, sucrose responsiveness and ability to discriminate odours through olfactory conditioning. Bees injected with insulin have higher spontaneous odour responses. Sucrose responsiveness and odour discrimination are differentially affected by treatment according to age: whereas insulin increases gustatory responsiveness and diminishes learning abilities of younger workers, it has the opposite effect on older bees. In summary, insulin can improve chemosensory responsiveness in young workers, but also worsens their learning abilities to discriminate odours. The insulin signalling pathway is responsive in young workers, although they are not yet initiating outdoor activities. Our results show strong age-dependent effects of insulin on appetitive behaviour, which uncover differences in insulin signalling regulation throughout the honeybee worker's adulthood.

KEY WORDS: *Apis mellifera*, Sucrose, Insulin, Chemosensory perception, Associative learning, Odour discrimination

INTRODUCTION

The honeybee *Apis mellifera* Linnaeus 1758 is a social insect that presents caste polyphenism (Wilson, 1971). Drones and queens constitute the reproductive castes whereas workers are sexually immature female individuals that perform collective tasks to maintain the nest's welfare. This caste exhibits age polyethism, which results in young adults performing duties inside the nest such as nursing, comb building, food processing, guarding or fanning. The oldest worker bees are foragers, who gather resources for the whole colony and transport it back to the hive (Lindauer, 1952; Seeley, 1982). Nutrition, age and reproductive status interact to regulate worker behaviour (Amdam et al., 2004). This temporal phenotypic progression is, in part, a consequence of physiological changes that the adult worker undergoes.

To explore these traits in honeybee workers, a hypothesis has been proposed. The reproductive ground plan hypothesis (Amdam et al., 2004; Hunt et al., 2007) suggests that temporal labour division is controlled by ovarian development (Turillazzi and West-Eberhard, 1996): nurses present rudimentary ovary development whereas foragers lose this feature (Amdam et al., 2004). These results lead to an integrative pathway where juvenile hormone (JH), vitellogenin (Vg) and insulin have pleiotropic effects on caste differentiation and worker labour division (Amdam et al., 2004; Ament et al., 2008; Lattorff and Moritz, 2013). JH, Vg and insulin levels differ between female castes (queen and workers), and vary throughout the adult bee's lifespan. JH titers show a slight increase from 2 to 4 days of the adult age and then remain extremely low until peak levels are reached when workers stop performing tasks within the nest and start foraging. By contrast, maximum Vg levels are found in young bees and then decrease until they become undetectable in foragers (Amdam et al., 2004; Hartfelder and Engels, 1998). Simultaneously, insulin signalling gene expression, including insulin-like peptides and their receptors, is higher in foragers than in nurses (Ament et al., 2008; Corona et al., 2007).

Nutrition has an important role in honeybee age polyethism (Amdam et al., 2007; Robinson, 1992). The onset age of foraging is affected by the colony's nutritional status (Amdam et al., 2007) and correlates with changes in the expression of genes implicated in feeding behaviour (Ament et al., 2008). Changes in nutritional status, which are regulated through the insulin signalling system, play a role in dictating behavioural shifts as workers age, which are likely to be mediated through social cues (Ament et al., 2008; Toth et al., 2005). Unlike vertebrates, expression of insulin-related genes in honeybees is negatively correlated with nutrient store: insulin levels increase whereas lipid stores decrease with worker age (Toth et al., 2005). From this, it can be postulated that insulin signalling relates to the forager's sensitivity to nutritional changes. The insulin-like peptide is mainly expressed in the brain and is upregulated in forager brains compared with those of nurses (Ament et al., 2008). This, in turn, would make foragers more sensitive to appetitive cues, contributing to their exaggerated responses to nutritional stimuli.

Honeybees exhibit an innate reflex towards antennal stimulation with sucrose solution, the proboscis extension response (PER). By assessing the PER towards water and sucrose solutions varying in concentration, it is possible to estimate the sucrose response threshold (SRT) of individuals, a measure of gustatory responsiveness (Page et al., 1998). This assay can give information about the bees' nutritional status (Martinez and Farina, 2008; Pankiw et al., 2004). Foragers often show high sucrose responsiveness, whereas the opposite is found in young and middle-aged bees (Scheiner et al., 2004). Regarding the effect on

[1]Laboratorio de Insectos Sociales, Departamento de Biodiversidad y Biología Experimental, IFIBYNE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Ciudad Universitaria, (C1428EHA) Buenos Aires, Argentina. [2]Université de Toulouse, UPS, Centre de Recherches sur la Cognition Animale, CNRS, F-31062, Toulouse Cedex 9, France.
*Present address: Department of Biological and Experimental Psychology, Queen Mary University of London, London, UK.
‡These authors contributed equally to this work

§Author for correspondence (walter@fbmc.fcen.uba.ar)

W.M.F., 0000-0001-6411-489X

Received 20 May 2016; Accepted 15 July 2016

Journal of Experimental Biology (2016) 219, 3003-3008 doi:10.1242/jeb.143511

List of abbreviations

AIC	Akaike's information criterion
CR	conditioned response
CS–	unrewarded conditioned stimulus
CS+	rewarded conditioned stimulus
DI	discrimination index
GLM	generalised linear model
GLMM	generalised linear mixed model
GRS	gustatory response score
JH	juvenile hormone
PER	proboscis extension response
QMP	queen mandibular pheromone
SOR	spontaneous odour response
SRT	sugar response threshold
Vg	vitellogenin

cognitive abilities, evidence suggests that a JH analogue affects short-term olfactory memory in recently emerged honeybees (Maleszka and Helliwell, 2001). JH positively influences the insulin-like signalling pathway in insects (Amdam and Seehuus, 2006; Corona et al., 2007; Hunt et al., 2007; Tu et al., 2005). Thus, we expect that insulin, with a strong age dependency, will not only affect sucrose and olfactory responsiveness but will also have an effect on learning abilities to discriminate odours in honeybees.

Apis mellifera has been considered a reference model within invertebrates to study behavioural and neural plasticity (Brown et al., 2004; Giurfa and Sandoz, 2012; Masson and Arnold, 1987; Menzel, 1999; Sigg et al., 1997; Winnington et al., 1996). In fact, at early ages of the adult stage, the central nervous system of honeybee workers completes its maturation (Masson and Arnold, 1987), and experiences undergone during this period can shape later physiology and behaviour (Arenas and Farina, 2008; Arenas et al., 2009a,b, 2012). Recently emerged and middle-aged workers show reliable learning performances in an olfactory PER conditioning (Mengoni Goñalons and Farina, 2015), similar to those of foragers. However, gustatory responsiveness and its sensitivity to environmental changes vary with age (Mengoni Goñalons and Farina, 2015; Ramírez et al., 2010).

Previous reports showed that endocrine secretions can tune chemosensory system in *Drosophila* (Ko et al., 2015; Root et al., 2011). However, these responses could differ strongly in social insects. Here, we assess the effect of insulin on appetitive behaviours such as chemosensory responsiveness and learning abilities in preforaging bees, especially in the case of young hive bees.

MATERIALS AND METHODS

Study site and animals

The study was carried out during the summer–autumn season of 2014 in the experimental field of the Facultad de Ciencias Exactas y Naturales of the Universidad de Buenos Aires, Argentina (34°32′S, 58°26′W). Newly emerged European honeybees (*A. mellifera*) were obtained from sealed brood frames taken from the experimental apiary and placed in an incubator at 36°C and 55% relative humidity. After emergence, workers were collected and reared in different environments: in the laboratory under controlled conditions or in an observational hive. For the former, emerging bees were collected in groups of up to 150 individuals and confined in wooden boxes (10×10×10 cm) with a metallic mesh on one side and a plastic door on other. Cages were kept in another incubator at 31°C. They offered 16% w/w sucrose solution and pollen *ad libitum*, and were checked every other day. Food was replaced every 2 days and dead bees were removed whenever needed (as previously described in Mengoni Goñalons and Farina, 2015). For hive rearing, emerging bees were marked with acrylic paint (ALBA-Argentina) on their thorax. A specific colour was used for each day of emergence so as to determine their age at a later stage. Marked bees were introduced into an observational hive that consisted of two brood frames, a mated queen and approximately 4000 workers, and were readily accepted by the rest of the colony (Breed et al., 2004). The hive was contained between acrylic walls that had a 40×25 cm window covered by a hinged door that allowed access to the colony. On the day of the experiment, marked bees were captured individually in plastic tubes and taken to the laboratory.

The experiments comply with the 'Principles of animal care', publication no. 86-23, revised 1985, of the National Institutes of Health, as well as with the current laws of the country in which the experiments were performed.

Experimental series and injections

With the purpose of assessing differential insulin effects according to adult age, four groups of pre-foraging bees were contemplated. Therefore, young bee workers were assessed when they were 2/3, 5/6, 9/10 or 14/15 days old.

Experimental bees were anaesthetised with ice and harnessed in carved pipette tips, which restrained body movement, but allowed them to freely move their mouthparts and antennae. Before they regained activity, they were injected with a microsyringe (NanoFil, World Precision Instruments) through the fourth and fifth segments of the abdomen. Treatment bees were injected with 1 µl of insulin (4 mg ml^{-1}, Human recombinant Zinc, Gibco, Grand Island, NY, USA) and control bees received 1 µl of Hepes buffer solution (25 mmol l^{-1}) (Ament et al., 2011). Solutions were used immediately after melting and kept in ice during the procedure. Twenty-four bees of the same age were used per injection session, which lasted approximately 45 min overall. In order to reduce effects resulting from differences in the time between injection and evaluation, treatment assignment was done by blocks, in which 12 bees were injected with insulin and the other 12 were injected with buffer solution. As two injection sessions were performed each day, the order in which the treatments were given in the morning was switched in the afternoon. The following day, the entire treatment assignment was alternated. All four age groups were represented in a pair of days. The order in which age groups were tested within each pair was randomised. Behavioural assays were performed 5 min after injection of the last bee. The order in which bees were injected was kept in the following procedure. Time between injection and the start of the assay was 25 min for all bees.

Spontaneous odour response

The harnessed bee was placed between a device that produced a constant airflow and an air extractor that removed released odours. The airstream (2.5 ml s^{-1}) was delivered to the head of the bee 2 cm away from it. An odour is considered a neutral stimulus and usually elicits no PER. Nevertheless, a naïve bee can still show a spontaneous response towards a certain odour. For this procedure, pure odours (Raguso and Pichersky, 1999), linalool and phenylacetaldehyde (Sigma-Aldrich, Steinheim, Germany), were used. Each was delivered for 6 s when, by means of an electric valve, the airflow was redirected to pass through a syringe containing 4 µl of the pure odour impregnated on a 30×3 mm filter paper. A spontaneous odour response (SOR) was considered when the bee fully extended its proboscis during odour delivery. Bees that responded to the

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 3003-3008 doi:10.1242/jeb.143511

mechanical air stimulus (16 s clean airflow before odour presentation) were discarded, as well as bees that did not respond to 50% w/w sucrose solution after the gustatory response assay (see following section). Odour presentations were 10 min apart, and the order was alternated from bee to bee (parts of this procedure were previously described in Mengoni Goñalons and Farina, 2015).

Gustatory responsiveness

Immediately after SOR evaluation, bees were stimulated with sucrose solutions of increasing concentrations (0.1, 0.3, 1, 3, 10, 30 and 50% w/w) by touching their antennae (Page et al., 1998). The lowest sucrose concentration at which an individual responded by extending its proboscis was interpreted as its SRT. Bees were lined up and tested sequentially for each concentration, i.e. all bees were presented with 0.1% solution first, then with 0.3% solution and so on. Before each sucrose solution presentation, all bees were tested for their response to water (0%). This controlled potential effects of repeated sucrose stimulation that could lead to increased sensitization or habituation, as well as ensuring that extension of the proboscis was not due to thirst. The inter-stimulus interval between water and sucrose solution was 4 min long. At the end of the experiment, a gustatory response score (GRS) was obtained for each bee. This score was based on the number of sucrose concentrations to which the bees responded. The response was arbitrarily quantified with scores from 1 to 7, where 1 represented a bee that only responded to the highest sucrose concentration, while a score of 7 represented an individual that responded to all concentrations tested. If a bee failed to respond to sucrose concentration in the middle of a response series (e.g. responded to 0.3, 3 and 10%, but did not respond to 1%), this 'failed' response was considered to be an error and the bee was deemed to have responded to that concentration as well. A bee that did not respond to any of the sucrose concentrations (score of 0) was excluded from further analyses. In addition, those bees that responded to all sucrose concentrations and all presentations of water were excluded from analyses as they appeared not to be able to discriminate between sucrose solution and water (as previously described in Mengoni Goñalons and Farina, 2015).

Odour discrimination in classical PER conditioning

For this procedure, only 5/6- and 14/15-day-old laboratory-reared bees were used. These naïve bees were presented with the same odours used in the SOR assay, but in this case linalool was paired with 50% w/w (rewarded conditioned stimulus, CS+; unconditioned stimulus) and phenylacetaldehyde was not (unrewarded conditioned stimulus, CS−). The harnessed bee was placed in the same context as described in the Spontaneous odour response section, above. During conditioning, odour was delivered for 6 s and, in the case of the CS+ presentation, the reward was presented during the last 3 s of this period by touching the antennae with 50% w/w sucrose solution and then feeding the bee. A conditioned response (CR) was considered to have occurred when the bee fully extended its proboscis during the first 3 s of odour delivery. One trial lasted for 39 s and was composed of 16 s of clean airflow, 6 s of odour and 17 s of clean airflow. Training consisted of five CS+ and five CS− trials arranged in a pseudo-randomised order (CS−, CS+, CS+, CS−, CS−, CS+, CS−, CS+, CS+, CS−). The inter-trial interval lasted approximately 15 min. To estimate the ability to discriminate between the two odours in the differential conditioning, we defined a global discrimination index (global DI) for each bee. In each trial pair, a bee was considered to have discriminated between the CS+ (linalool) and the CS− (phenylacetaldehyde) only if it extended its proboscis towards the first, but not the latter. The global DI was calculated as the number of trial pairs the bee succeeded in discriminating between the two odours (values include 0 through 4).

A period of 20 min elapsed between the last trial and the testing phase. The latter consisted of non-rewarded presentations of the CS+ and the CS−, alternating their order from bee to bee. After the testing phase, the response to 50% w/w sucrose solution was verified and only responding bees were taken into account (a similar procedure was previously described in Mengoni Goñalons and Farina, 2015).

Statistical analysis

The effects of factors on all variables were assessed by means of generalised linear models (GLM) or generalised linear mixed models (GLMM). Models were fitted in R (R Foundation for Statistical Computing, Vienna, Austria) using the glm function for the former case and the glmer function of the lme4 package (Bates et al., 2015) for the latter. Alternative models were assessed and compared, and one was chosen depending on its parsimony and its Akaike's information criterion (AIC) value. *Post hoc* comparisons were performed with the glht function of the R package multcomp (Hothorn et al., 2008).

An SOR was defined as the extension of the proboscis towards any of the two odours. Effect of insulin injection on SOR was assessed by means of a GLM with binomial error structure. The initial model included rearing environment, age and treatment as fixed factors. In addition, to test for any odour bias, spontaneous responses to each odour were considered in a separate analysis. In this case, a GLMM was used and the initial model included rearing environment, age, treatment and odour as fixed factors and subject bee as a random factor. Gustatory responsiveness was estimated through the GRS, which is a sum of the unconditioned responses to the sugar solutions presented in the procedure. Values include 1 through 7. The effect of insulin injection on GRS was assessed by means of a GLM with binomial error structure. The initial model included rearing environment, age and treatment as fixed factors.

A bee that extended its proboscis towards odours in the first trial pair was considered to show a spontaneous response and was not taken into account for conditioning analysis. The effect of insulin on olfactory discrimination was assessed by means of a GLM with binomial error structure. The initial model included treatment and age as fixed factors. In the testing phase, no bee extended its proboscis towards the CS−. Therefore, effect of insulin injection was only studied on CR towards the CS+ and assessed by means of a GLM that included treatment and age as fixed factors.

RESULTS

Spontaneous odour response

Bees were presented with two odour stimuli (linalool and phenylacetaldehyde). We defined a spontaneous response as the extension of the proboscis towards any of the two odours. Insulin-injected bees, independently of age or rearing environment, had higher SOR than control bees. As the minimum model did not include these factors, rearing environment or age had no effect on SOR (SOR ~ treatment, AIC=625.13, Z=2.585, P=0.0097; Fig. 1; Table S1). In addition, there was no bias towards a certain stimuli (SOR ~ odour+bee, Z=0.00, P=1; Table S2). Therefore, we can state that there was no odour preference.

Gustatory responsiveness

Immediately after SOR evaluation, bees were presented with increasing concentrations of sucrose solutions. A GRS was defined as the sum of positive responses throughout the procedure

Journal of Experimental Biology (2016) 219, 3003-3008 doi:10.1242/jeb.143511

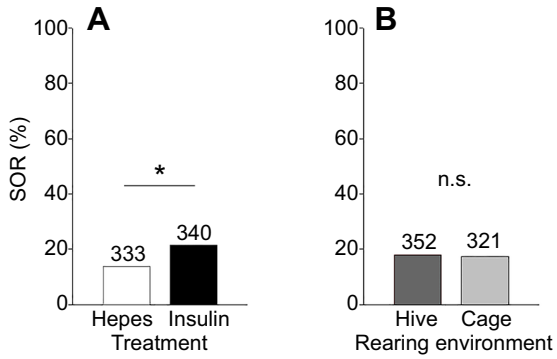

Fig. 1. Insulin injection increased the probability of spontaneous odour response, independently of age or rearing environment, in the honeybee *Apis mellifera*. Percentage of bees that extended their proboscis towards either of the two odours, linalool and phenylacethaldehyde (spontaneous odour response, SOR). Responses of bees of all ages (A) after being injected with Hepes buffer (white) or insulin (black) and (B) obtained from an experimental hive (dark grey) or from cages kept in the laboratory (light grey). Minimal adequate model: SOR ~ treatment. Asterisk indicates a significant difference (P<0.05); n.s., not significant. Values above bars indicate the number of bees tested.

(Page et al., 1998). Hive-reared bees had lower GRS than laboratory-reared bees (GRS ~ rearing environment+age×treatment, AIC=2083.1, Z=5.497, P<0.001; Fig. 2A; Table S3). This effect was independent of age and treatment, as models containing interactions between rearing environment and these two factors had higher AIC values than the one chosen. Effect of insulin on gustatory responsiveness depended on age of injection. Treated 2/3-day-old bees presented higher GRS than control bees (Z=4.685, P<0.001), which means their SRT was lower. On the contrary, insulin injection of 14/15-day-old bees had a depressing effect on GRS, raising their SRT (Z=−4.375, P<0.001). Bees 5 to 10 days old were not affected by insulin (5/6 days old: Z=0.890, P=0.8080; 9/10 days old: Z=−1.521, P=0.3770; Fig. 2B).

Odour discrimination in classical PER conditioning

For this procedure, 5/6- and 14/15-day-old laboratory-reared bees were used. A global DI was defined for each bee as the number of trial pairs the bee succeeded in discriminating between the two odours (if it extended its proboscis towards the CS+, but not the CS−). An effect of insulin was observed in bees of both ages (global DI ~ age×treatment, AIC=576.84; Table S4). Treated 5/6-day-old bees presented lower global DI than control bees. On the contrary, insulin injection of 14/15-day-old bees had an increasing effect on global DI (5/6 days old: Z=−3.493, P<0.001; 14/15 days old: Z=4.919, P<0.001; Fig. 3A). In addition, control older bees showed lower global DI than control younger bees (Z=4.428, P<0.001; Fig. 3A, white bars). In the testing phase, injection of insulin had a similar effect on CR to the CS+ (CR ~ age×treatment, AIC=206.66; Fig. 3B; Table S5). Insulin reduced CR in younger bees, but raised it in older bees (5/6 days old: Z=−2.349, P=0.0383; 14/15 days old: Z=2.399, P=0.0341; Fig. 3B). In addition, control older bees showed lower conditioned responses than control younger bees (Z=2.829, P=0.0103; Fig. 3B, white bars).

DISCUSSION

Our study reveals that insulin improves spontaneous response to odours independent of rearing environment and age. In addition, whereas it improves sucrose responsiveness and reduces odour discrimination in younger honeybees, it has the opposite effect in older ones. We expected insulin to have an effect on gustatory responsiveness in younger workers and little or no effect in older ones. The results partially verify our prediction, as 2/3-day-old bees were affected by insulin injection, but 5/6- and 9/10-day-old bees were not. Given that insulin levels increase with worker age (Corona et al., 2007), these results indicate that exogenous insulin artificially induces the youngest workers to exhibit higher responsiveness to sucrose, a trait associated with foragers (Scheiner et al., 2004). In addition, the youngest bees also increased their probability to respond spontaneously to odours after treatment administration. To sum up, insulin injection improves chemosensory responsiveness. This seems to be an adaptive function in terms of division of labour, as nurse bees would be able to modify their chemosensory thresholds, which is required when social structure undergoes a change as a result of a selective pressure, such as the sudden death of a portion of the foragers. In this case, nurses and food processors are forced to perform outside tasks at a younger age than expected. These precocious bees exhibit a decrease in Vg levels and lipid reserves in the hemolymph and an increase in JH and insulin levels (Corona et al., 2007; Hartfelder and Engels, 1998), and they show a poor foraging performance (Chang et al., 2015; Perry et al., 2015). Similarly, pollen foragers, who begin outside tasks earlier than nectar foragers, show high gustatory responsiveness, a quality that indicates a poor nectar foraging ability (Amdam et al., 2006). This suggests that young hive bees that are artificially induced to becoming foragers through a rise in insulin levels would be poor nectar gatherers.

Contrary to what was expected, our results indicate that 14/15-day-old treated bees had lower GRS values than control bees. Thus, the biological effect found was the inverse of that found in the youngest bees. On top of the high insulin levels that would be found in these

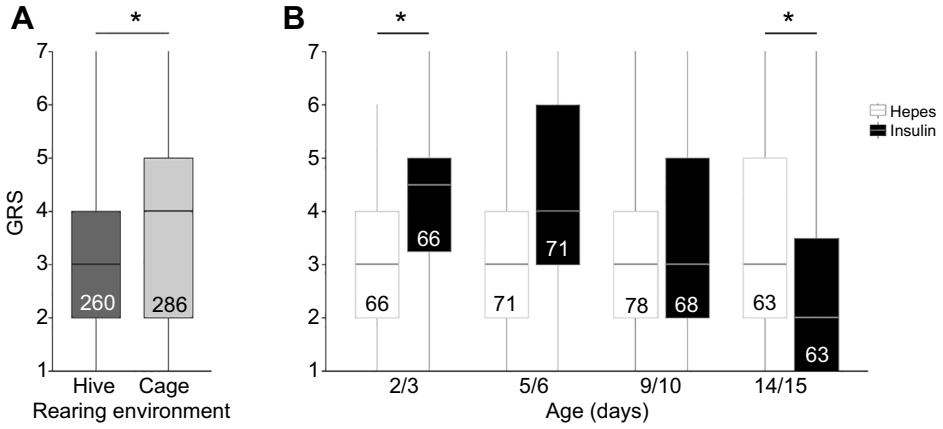

Fig. 2. Effect of insulin injection on gustatory responsiveness was age dependent in *A. mellifera*. Gustatory response scores (GRS) of (A) bees obtained from an observational hive (dark grey) or from cages kept in the laboratory (light grey) and (B) 2/3-, 5/6-, 9/10- and 14/15-day-old bees after being injected with Hepes buffer (white) or insulin (black). Minimal adequate model: GRS ~ rearing environment+age×treatment. The thick line, box and whiskers represent the median, interquartile interval and data range, respectively. Asterisks indicate for statistical differences between injection treatments within an age group (P<0.05). Values in bars indicate the number of bees tested.

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 3003-3008 doi:10.1242/jeb.143511

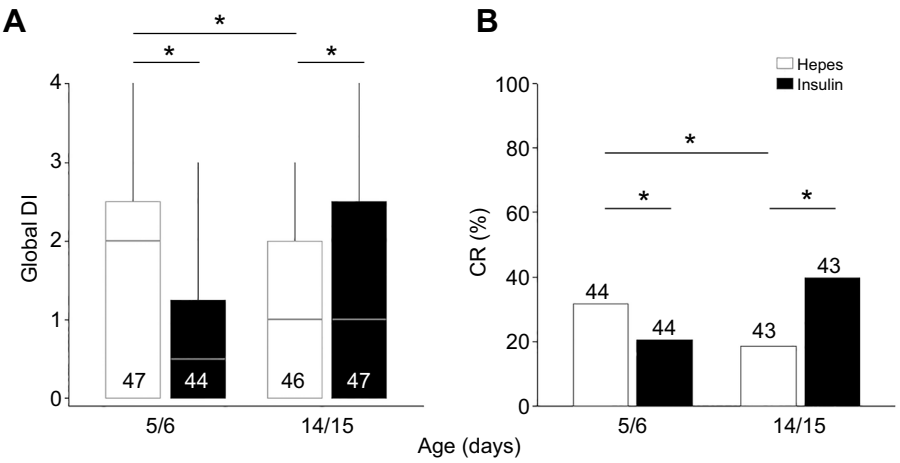

Fig. 3. Effect of insulin injection on odour discrimination in PER conditioning of *A. mellifera* was age dependent. (A) Global discrimination indices (global DI) during acquisition and (B) percentage of bees that extended their proboscis towards the rewarded conditioned stimulus (conditioned response, CR) during the testing phase performed 20 min after acquisition, after being injected with Hepes buffer (white) or insulin (black). Bees were 5/6 and 14/15 days old when assessed and reared under laboratory conditions. Minimal adequate models: global DI ~ age×treatment, CR ~ age×treatment. In A, the thick line, box and whiskers represent the median, inter-quartile interval and data range, respectively. Asterisks indicate for statistical differences between injection treatments within an age group (P<0.05). Values in or above bars indicate the number of bees tested.

middle-aged bees, an additional insulin shock did not increase sucrose responsiveness, but decreased it. Sucrose responsiveness is closely related to nutritional status and, thus, feeding motivation (Martinez and Farina, 2008; Pankiw et al., 2004). As high levels of insulin lead to foraging activities (Ament et al., 2008), it seems clear that the foraging task is directly tuned by insulin levels present in the bee hemolymph. Despite the fact that we did not include actual foragers in our study, we claim that the 14/15-day-old bees used in our experiments were in a corresponding physiological state to foragers, as their coetaneous sisters – emerged as adults on the same day – were seen flying to an artificial feeder 2 days later. A honeybee forager does not seek food for individual sustenance, but instead contributes to colony reserves. Nonetheless, it still needs motivation to initiate a foraging trip. In other words, insulin would be comparatively low when the forager is in the hive, allowing it to eat. Once the forager is satiated, insulin levels rise until reaching a threshold that would trigger a foraging flight (Ament et al., 2008). This fine insulin regulation would then explain why insulin-injected 14/15-day-old bees show lower GRS compared with control bees.

Laboratory-reared bees had higher gustatory responsiveness than hive bees. These results are not surprising as bees reared in the hive had been exposed to the queen mandibular pheromone (QMP), which is released by the queen, and QMP reduces sucrose responsiveness (Pankiw and Page, 2003). In addition, laboratory bees exposed to QMP, which simulates queen presence, has no effect on the expression genes involved in the insulin signalling peptide (Fischer and Grozinger, 2008). This last result corroborates our own findings that the effect of insulin did not depend on whether workers were reared in the hive or queen deprived in the laboratory.

Insulin also affected olfactory learning performance. In younger workers, insulin improved chemosensory responsiveness but worsened olfactory discrimination. Therefore, contrary to our expectations, higher gustatory responsiveness as a result of insulin injection did not result in better cognitive abilities in terms of odour discrimination (Mengoni Goñalons and Farina, 2015; Ramírez et al., 2010; Scheiner et al., 2004). In fact, the correlation was the opposite. It appears that the adaptive function of modifying gustatory responsiveness suggested earlier does not apply to olfactory learning. In this case, young bees forced to collect resources would become foragers, but bad learners in terms of discriminating floral odours. This olfactory discrimination is beneficial when selecting foraging sites, but is not essential if the colony is facing a drastic change in social structure. Therefore, having all the endocrine systems – including the insulin system – ready for the transition from young hive bees to foragers is valuable but it also introduces penalties in terms of foraging efficiency. In addition, we found contrasting effects of insulin on both olfactory learning and gustatory responsiveness between young and middle-aged workers.

Ultimately, the same results were observed in both acquisition and testing phases of the olfactory conditioning, which implies that insulin modifies the bee's chemosensory responsiveness, and this modification lasts at least 20 min after conditioning, suggesting an effect on medium-term memory.

Vertebrate insulin (bovine: Mott and Breed, 2012; human: present study) has been shown to be bioreactive in honeybees. In our case, the mode of administration was systemic and general, and we assessed variables in a short-term period. Therefore, it is not possible to elucidate the hormone's targets and the source of its behavioural effects. We infer that the insulin pathway is a key to understanding how the different metabolic pathways act in concert to synchronise the development of chemosensory and physiological processes. Many questions remain unanswered about what other pathways are involved after an artificial rise in insulin level in the hemolymph of honeybees. However, this study reveals one of the main roles of insulin in adult honeybee development and provides tools for research on how insulin affects labour division in bees as well as a worker's individual physiological state.

Acknowledgements
We also thank K. Lukowiak and two anonymous referees for their valuable comments and suggestions on an earlier version of this manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
C.M.G., M.G., M.G.d.B.S. and W.M.F. conceived and designed the experiments. C.M.G. and M.G. performed the experiments. C.M.G. performed data analysis. C.M.G., M.G., M.G.d.B.S. and W.M.F. drafted the manuscript. All authors revised and commented on the manuscript.

Funding
C.M.G. and W.M.F. thank the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) and the Universidad de Buenos Aires (UBA) for support. This study was partly supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT 2013 1060), UBA (20020130100185BA) and CONICET (PIP 112-201501-00633) to W.M.F. M.G. and M.G.d.B.S. thank the Centre de Recherches sur la Cognition Animale and the University Paul Sabatier (grant APIGENE) for support.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.143511.supplemental

References

Amdam, G. V. and Seehuus, S.-C. (2006). Order, disorder, death: lessons from a superorganism. *Adv. Cancer Res.* **95**, 31-60.

Amdam, G. V., Norberg, K., Fondrk, M. K. and Page, R. E. (2004). Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. *Proc. Natl. Acad. Sci. USA* **101**, 11350-11355.

Amdam, G. V., Norberg, K., Page, R. E., Jr., Erber, J. and Scheiner, R. (2006). Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). *Behav. Brain Res.* **169**, 201-205.

Amdam, G. V., Nilsen, K.-A., Norberg, K., Fondrk, M. K. and Hartfelder, K. (2007). Variation in endocrine signaling underlies variation in social life history. *Am. Nat.* **170**, 37-46.

Ament, S. A., Corona, M., Pollock, H. S. and Robinson, G. E. (2008). Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc. Natl. Acad. Sci. USA* **105**, 4226-4231.

Ament, S. A., Velarde, R. A., Kolodkin, M. H., Moyse, D. and Robinson, G. E. (2011). Neuropeptide Y-like signalling and nutritionally mediated gene expression and behaviour in the honey bee. *Insect Mol. Biol.* **20**, 335-345.

Arenas, A. and Farina, W. M. (2008). Age and rearing environment interact in the retention of early olfactory memories in honeybees. *J. Comp. Physiol. A* **194**, 629-640.

Arenas, A., Fernández, V. M. and Farina, W. M. (2009a). Associative learning during early adulthood enhances later memory retention in honeybees. *PLoS ONE* **4**, e8046.

Arenas, A., Giurfa, M., Farina, W. M. and Sandoz, J. C. (2009b). Early olfactory experience modifies neural activity in the antennal lobe of a social insect at the adult stage. *Eur. J. Neurosci.* **30**, 1498-1508.

Arenas, A., Giurfa, M., Sandoz, J. C., Hourcade, B., Devaud, J. M. and Farina, W. M. (2012). Early olfactory experience induces structural changes in the primary olfactory center of an insect brain. *Eur. J. Neurosci.* **35**, 682-690.

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R. H. B., Singmann, H., Dai, B., Grothendieck, G., Eigen, C. and Rcpp, L. (2015). Package 'lme4'. *Convergence* **12**, 1.

Breed, M. D., Diaz, P. H. and Lucero, K. D. (2004). Olfactory information processing in honeybee, *Apis mellifera*, nestmate recognition. *Anim. Behav.* **68**, 921-928.

Brown, S. M., Napper, R. M. and Mercer, A. R. (2004). Foraging experience, glomerulus volume, and synapse number: a stereological study of the honey bee antennal lobe. *J. Neurobiol.* **60**, 40-50.

Chang, L.-H., Barron, A. B. and Cheng, K. (2015). Effects of the juvenile hormone analogue methoprene on rate of behavioural development, foraging performance and navigation in honey bees (*Apis mellifera*). *J. Exp. Biol.* **218**, 1715-1724.

Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. A. and Robinson, G. E. (2007). Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc. Natl. Acad. Sci. USA* **104**, 7128-7133.

Fischer, P. and Grozinger, C. M. (2008). Pheromonal regulation of starvation resistance in honey bee workers (*Apis mellifera*). *Naturwissenschaften* **95**, 723-729.

Giurfa, M. and Sandoz, J.-C. (2012). Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* **19**, 54-66.

Hartfelder, K. and Engels, W. (1998). Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. *Curr. Top. Dev. Biol.* **40**, 45-77.

Hothorn, T., Bretz, F., Westfall, P. and Heiberger, R. M. (2008). *Multcomp: Simultaneous Inference in General Parametric Models. R package version 1.0-0*. Vienna, Austria: R Foundation for Statistical Computing.

Hunt, G. J., Amdam, G. V., Schlipalius, D., Emore, C., Sardesai, N., Williams, C. E., Rueppell, O., Guzmán-Novoa, E., Arechavaleta-Velasco, M., Chandra, S. et al. (2007). Behavioral genomics of honeybee foraging and nest defense. *Naturwissenschaften* **94**, 247-267.

Ko, K. I., Root, C. M., Lindsay, S. A., Zaninovich, O. A., Shepherd, A. K., Wasserman, S. A., Kim, S. M. and Wang, J. W. (2015). Starvation promotes concerted modulation of appetitive olfactory behavior via parallel neuromodulatory circuits. *eLife* **4**, e08298.

Lattorff, H. M. G. and Moritz, R. F. (2013). Genetic underpinnings of division of labor in the honeybee (*Apis mellifera*). *Trends Genet.* **29**, 641-648.

Lindauer, M. (1952). Ein beitrag zur frage der arbeitsteilung im bienenstaat. *Z. Vgl. Physiol.* **34**, 299-345.

Maleszka, R. and Helliwell, P. (2001). Effect of juvenile hormone on short-term olfactory memory in young honeybees (*Apis mellifera*). *Horm. Behav.* **40**, 403-408.

Martinez, A. and Farina, W. M. (2008). Honeybees modify gustatory responsiveness after receiving nectar from foragers within the hive. *Behav. Ecol. Sociobiol.* **62**, 529-535.

Masson, C. and Arnold, G. (1987). Organization and plasticity of the olfactory system of the honeybee, Apis mellifera. In *Neurobiology and Behavior of Honeybees* (ed. R. Menzel and A. Mercer), pp. 280-295. Berlin: Springer Verlag.

Mengoni Goñalons, C. and Farina, W. M. (2015). Effects of sublethal doses of imidacloprid on young adult honeybee behaviour. *PLoS ONE* **10**, e0140814.

Menzel, R. (1999). Memory dynamics in the honeybee. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* **185**, 323-340.

Mott, C. M. and Breed, M. D. (2012). Insulin modifies honeybee worker behavior. *Insects* **3**, 1084-1092.

Page, R., Jr., Erber, J. and Fondrk, M. (1998). The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* **182**, 489-500.

Pankiw, T. and Page, R. Jr. (2003). Effect of pheromones, hormones, and handling on sucrose response thresholds of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* **189**, 675-684.

Pankiw, T., Nelson, M., Page, R. E., Jr. and Fondrk, M. K. (2004). The communal crop: modulation of sucrose response thresholds of pre-foraging honey bees with incoming nectar quality. *Behav. Ecol. Sociobiol.* **55**, 286-292.

Perry, C. J., Søvik, E., Myerscough, M. R. and Barron, A. B. (2015). Rapid behavioral maturation accelerates failure of stressed honey bee colonies. *Proc. Natl. Acad. Sci. USA* **112**, 3427-3432.

Raguso, R. A. and Pichersky, E. (1999). New perspectives in pollination biology: floral fragrances. A day in the life of a linalool molecule: chemical communication in a plant-pollinator system. Part 1: Linalool biosynthesis in flowering plants. *Plant Species Biol.* **14**, 95-120.

Ramírez, G. P., Martínez, A. S., Fernández, V. M., Bielsa, G. C. and Farina, W. M. (2010). The influence of gustatory and olfactory experiences on responsiveness to reward in the honeybee. *PLoS ONE* **5**, e13498.

Robinson, G. E. (1992). Regulation of division of labor in insect societies. *Annu. Rev. Entomol.* **37**, 637-665.

Root, C. M., Ko, K. I., Jafari, A. and Wang, J. W. (2011). Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. *Cell* **145**, 133-144.

Scheiner, R., Page, R. E. and Erber, J. (2004). Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). *Apidologie* **35**, 133-142.

Seeley, T. D. (1982). Adaptive significance of the age polyethism schedule in honeybee colonies. *Behav. Ecol. Sociobiol.* **11**, 287-293.

Sigg, D., Thompson, C. M. and Mercer, A. R. (1997). Activity-dependent changes to the brain and behavior of the honey bee, *Apis mellifera* (L.). *J. Neurosci.* **17**, 7148-7156.

Toth, A. L., Kantarovich, S., Meisel, A. F. and Robinson, G. E. (2005). Nutritional status influences socially regulated foraging ontogeny in honey bees. *J. Exp. Biol.* **208**, 4641-4649.

Tu, M.-P., Yin, C.-M. and Tatar, M. (2005). Mutations in insulin signaling pathway alter juvenile hormone synthesis in *Drosophila melanogaster*. *Gen. Comp. Endocrinol.* **142**, 347-356.

Turillazzi, S. and West-Eberhard, M. J. (ed.) (1996). Natural history and evolution of paper wasps. Oxford: Oxford University Press.

Wilson, E. O. (1971). *The Insect Societies*. Cambridge, MA: Belknap Press.

Winnington, A. P., Napper, R. M. and Mercer, A. R. (1996). Structural plasticity of identified glomeruli in the antennal lobes of the adult worker honey bee. *J. Comp. Neurol.* **365**, 479-490.