



Short communication

Insect psychoneuroimmunology: Immune response reduces learning in protein starved bumblebees (*Bombus terrestris*)

Carolyn E. Riddell, Eamonn B. Mallon *

Department of Biology, University of Leicester, UK

Received 10 May 2005; received in revised form 27 June 2005; accepted 27 June 2005

Abstract

It is well established in vertebrates that there are many intricate interactions between the immune system and the nervous system. Here, we present behavioural evidence indicating a link between the immune system and the nervous system in insects. We show that otherwise non-infected bumblebees whose immune systems were challenged by a non-pathogenic immunogenic elicitor (lipopolysaccharide) have reduced abilities to learn or recall a memory in a classical conditioning paradigm. There is evidence that protein is intricately involved as this immune induced reduction in memory only becomes apparent after the bees are deprived of pollen (their only protein sources).

© 2005 Elsevier Inc. All rights reserved.

Keywords: Octopamine; Eicosanoids; Gram negative bacteria; Associative learning; Proboscis extension

1. Introduction

One important recent advance in understanding the biological basis of behaviour is the recognition that there is extensive communication between the central nervous system and the immune system. A healthy individual has been defined as one whose immune system remains quiet and does not interfere with brain processing of external information (Dantzer, 2004). For example, it has been discovered that many behavioural responses to infectious agents, such as fever, increased slow-wave sleep, reduced activity, exploration, and sexual behaviour are orchestrated by immune products called proinflammatory cytokines that are released in response to the detection of antigens (Maier and Watkins, 1998). Another example of the reach of psychoneuroimmunology is that although pain is obviously centrally mediated, only recently has the pain associated with inflammation been

targeted by drugs acting at the levels of cytokines, that are produced and exert their effects within the central nervous system (Maier, 2003).

Recently, it has been shown that links between nervous and immune systems are not unique to vertebrates. The honeybee *Apis mellifera* was shown to have reduced learning abilities when its immune system had been stimulated non-pathogenically (Mallon et al., 2003). The aims of the current study are to generalise this result by looking for this immune reduced memory function in the bumblebee *Bombus terrestris*, and to begin to unravel the physiological basis of this phenomenon.

Associative learning and memory formation, in the bumblebee can be readily studied and quantified by exploiting the proboscis extension reaction (PER) (Laloi et al., 1999). In this experimental paradigm, when their antennae are stimulated by sugar water (the unconditioned stimulus) bumblebees extend their proboscis. If, prior to this sugar stimulation an odour (the conditioned stimulus) is presented, the bumblebee will associate this odour with the sugar stimulus. The extent to which the bee learns can then be quantified by presenting the

* Corresponding author. Fax: +44 116 2528333.
E-mail address: ebm3@le.ac.uk (E.B. Mallon).

conditioned stimulus alone and recording if the bee responds or not.

A possible mechanism by which immune response could affect learning is through a trade-off for some substance required by both systems. The immune system depletes the pool of this substance while fighting our phantom infection leaving insufficient resources for the nervous system to form/recall memories properly. One among many possible candidate trade-off substance could be octopamine, a biogenic amine. In honeybees, there is strong evidence that associative learning depends on the release of octopamine (Mueller, 2000). It has also been demonstrated to be important in the insect immune response (Wiesner et al., 1996). Alternatively, some as of yet unidentified product of the immune system could somehow inhibit the nervous system. As an example, eicosanoids (Stanley, 2000), oxygenated metabolites of certain poly-saturated fatty acids (arachidonic acid), act as mediators of insect responses to bacterial infection (as simulated here by the LPS-challenge) (Dean et al., 2002). They also have been shown to modulate invertebrate neural and synaptic physiology (Piomelli, 1994).

An attempt to replicate the honeybee result (Brockmann, pers commun.) showed no difference between immune challenged and control honeybees in memory abilities. Further analysis suggested that this was due to the bees in the original experiment (Mallon et al., 2003) being under nutritional stress. Previously, it has been found that effects of the immune response only became apparent when bees were starved (Moret and Schmid-Hempel, 2000). For both our hypotheses (Octopamine, Eicosanoids), we would expect protein consumption to increase to compensate for the effect of the immune response. Octopamine is a biogenic amine requiring protein for its synthesis, so as it becomes depleted, bees should increase consumption of protein. Protein is not required for immune induced eicosanoid synthesis since it is lipid based. Rather protein would be probably required to ameliorate the disruptive effects of eicosanoids released during an infection. Recent work has shown that insects possess a well developed ability to control the balance of macronutrients while foraging (Mayntz et al., 2005).

2. Methods

2.1. Injection of treatments

We challenged the bee's immune system by injecting, into the haemolymph, a dose of 5 μ l of Ringer solution containing 4% lipopolysaccharide (LPS, Sigma L-2755) (0.5 mg/ml = 9 mg 4% LPS/g of bee), a highly immunogenic but non-pathogenic elicitor of the immune response (Moret and Schmid-Hempel, 2000). An experimental group of 62 bees were injected with LPS. To con-

trol for injection another set of bees ($n=55$) were injected just with 5 μ l of Ringer solution, a saline solution regularly used in insect physiology. The bees originated from two different colonies and were assigned such as to balance colony origin across all treatments. After experimental manipulation, each group was housed for one week in plastic containers (17 \times 13.5 \times 9.5 cm). Some bees were fed ad libitum on both sugar water and fresh frozen pollen ($n=57$). The rest were fed sugar water only ($n=60$).

2.2. Proboscis extension reflex assay

Six hours before they were to be tested, bees were harnessed in small metal tubes. They were fed ad libitum with sugar water and then not fed until the assay began. Bees were checked to see did they extend their proboscis just to the sugar solution. The two groups had similar levels of response (Ringer 75%, LPS 72%). Bees that did not respond were excluded. The olfactory conditioned stimulus used was a 10% solution of Linalool (Sigma; 95–97%) in hexane. Each bee was given 10 conditioned stimulus/unconditioned stimulus pairings. For this, each bee was first exposed to a continuous air stream for 15 s. Then, the linalool odour (CS) was pulsed into the continuous air stream for 12 s. For the last 6 s of this, the antennae were stimulated with 75% sucrose water (US). If the bee responded during the final 6 s, it was rewarded with sugar water. During the first 6 s of CS (when the odour was presented by itself), the bee was observed for a proboscis extension. If a spontaneous PER occurred on the first pairing, this bee was not used again. The time between two subsequent CS–US pairings was 15 min.

2.3. Statistical analysis

The method of generalized estimating equations (GEE) was used to account for correlations among observations from the same subject in a repeated measures logistic regression (Hardin and Hilbe, 2003). The GEE method estimates the regression parameters assuming that the observations are independent, uses the residuals from this model to estimate the correlations among observations from the same subjects, and then uses the correlation estimates to obtain new estimates of the regression parameters. This process is repeated until the change between two successive estimates is very small. GEE was implemented in Intercooled STATA 8.2 (using the XTGEE procedure).

3. Results

There was no significant difference between treatments in the number of responses the bees made to the unconditioned stimulus throughout the 10 trials (pollen

fed: $z = -0.57$, $n = 470$, $p = .571$; no pollen fed: $z = 1.6$, $n = 598$, $p = .109$, Fig. 1).

When bees were fed pollen during the 1st week refractory period after the injection, there was no significant difference between treatments in their response to the conditioned stimulus alone ($z = 0.73$, $n = 416$, $p = .466$, Fig. 2A). However, when the bees were starved of pollen, but still received plenty of sugar water, LPS injected bees were found to have a much lower probability of responding compared to the control ringer injected bees ($z = -3.24$, $n = 513$, $p = .001$, Fig. 2B).

There was no difference between the pollen-starved ringer injected and the pollen fed ringer injected bees ($z = 1.65$, $n = 460$, $p = .1$). However, LPS injected bees showed a reduction in response levels if they were deprived of pollen ($z = 2.41$, $n = 469$, $p = .016$). In none of our comparisons was there a significant colony origin effect.

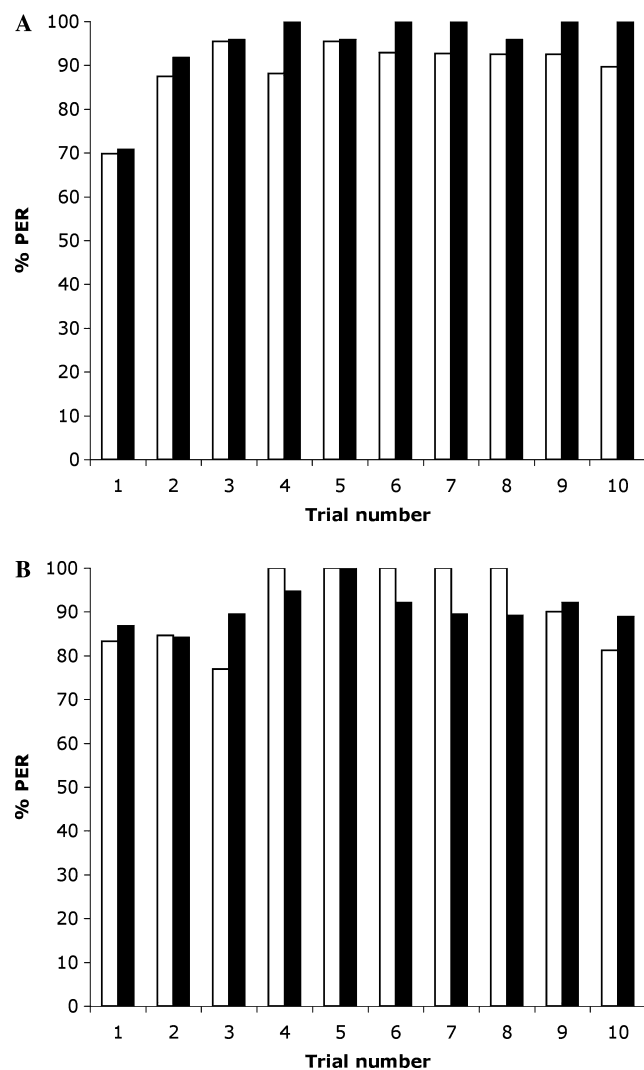


Fig. 1. Percentage of pollen fed bees (A) or protein starved bees (B) showing a proboscis extension reflex to the unconditioned stimulus across 10 learning trials. White bars represent bees injected with ringer (unfed = 33, fed = 19) and the black bars are the responses of LPS injected bees (unfed = 21, fed = 41).

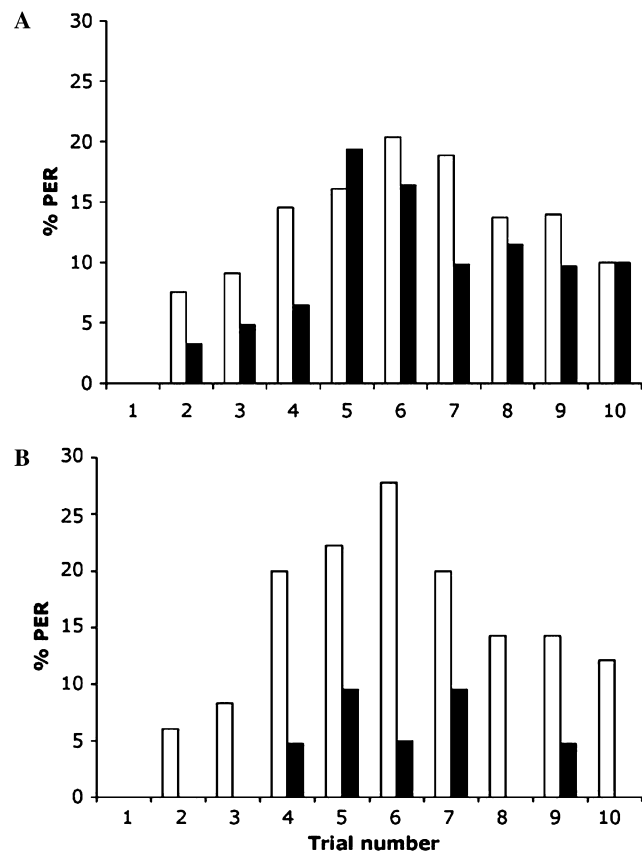


Fig. 2. Percentage of pollen fed bees (A) or protein starved bees (B) showing a proboscis extension reflex to the conditioned stimulus across 10 learning trials. White bars represent bees injected with ringer (unfed = 33, fed = 19) and the black bars are the responses of LPS injected bees (unfed = 21, fed = 41). The peaking of responses at trial six and its subsequent decline is most likely due to the bees' motivation to learn being reduced due to decreased hunger. At each previous trial, the bees would have taken a small amount of sugar water, over the course of six or so trials this would reduce the motivation of the bees to respond to further stimuli. This decrease in motivation to learn would happen much earlier compared to the bee actually being sated, when the result would be seen in Fig. 1.

4. Discussion

In this study, we present evidence that bumblebees, whose immune systems were stimulated non-pathogenically, perform poorly in a memory test. This result only became obvious when they were deprived of a protein source (pollen).

Recently it has become evident that worker bees need protein throughout their adult lifespan (Smeets and Duchateau, 2003). Here, we show that bumblebees require protein to ameliorate the effect of the immune response on their learning abilities. When fed a protein-rich diet, the ability to respond to a conditioned stimulus is not significantly reduced when the immune system was challenged (although it does show the similar decreasing trend Fig. 1A). The protein deficient diet did not simply impair

ability to perform PER generally, since ringer treated bees were unaffected by the feeding adjustment. It could also not have been simply due to an energy trade-off as all bees were fed unlimited amounts of sugar solution. Although, we cannot currently rule out the idea that the nutritional imbalance induces starvation stress, which then effects brain physiology. It seems unlikely to us that our result is due simply to this interaction between condition and treatment, i.e., the LPS insult is too much for animals in poor condition (pollen starved). If this were the case, we might expect that the response to the unconditioned stimulus (a measure of general activity) to be affected by treatment and feeding protocol. It was not (Fig. 1). Whether protein is used to compensate for a trade-off (e.g., octopamine) or to repair damage caused (eicosanoid induced) is not yet clear.

However, the eicosanoid damage hypothesis offers tantalizing links to vertebrate psychoneuroimmunology. It has been shown in rats, that a similar immune induced memory reduction is due to the effects of interleukin 1- β (IL-1 β) (Pugh et al., 2001). IL-1 β is a proinflammatory cytokine known to play a role in the 'sickness syndrome' (Maier and Watkins, 1998). Peripheral injection of LPS in rats increases IL-1 β levels and decreases memory consolidation (Pugh et al., 1999). If an IL-1 β receptor antagonist had been administered previously, memory consolidation is not reduced. Future work on insect immunity involves replicating this result with, for example, dexamethasone, a specific inhibitor of the eicosanoid pathway (Stanley-Samuelson et al., 1991). Eicosanoids are of course not the only possible signalling molecules. It is possible that cross-talk between the immune and nervous system in invertebrates may involve cytokines. However, the study of cytokines in invertebrates is still in its infancy (Gillespie et al., 1997).

We have shown that immune system–neural system interactions are not unique to vertebrates. Rather they have now been found in two separate species of insects. Although there is much functional difference between insects and vertebrates, with appropriate models, insects can help illustrate systems in higher animals. There is much to be discovered about the nature of cross-talk between the insect immune and nervous systems. Will its molecular basis be similar to the vertebrate system? This study demonstrates the utility of an invertebrate model and opens the way for a new area of study, using invertebrates as models for psychoneuroimmunology.

Acknowledgments

The authors thank Sally Adams and Tom Matheson and three anonymous referees for helpful comments. This work was funded by an ASAB grant to E.B.M.

References

- Dantzer, R., 2004. Innate immunity at the forefront of psychoneuroimmunology. *Brain Behavior and Immunity* 18, 1–6.
- Dean, P., Gadsden, J.C., Richards, E.H., Edwards, J.P., Charnley, A.K., Reynolds, S.E., 2002. Modulation by eicosanoid biosynthesis inhibitors of immune responses by the insect *Manduca sexta* to the pathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* 79, 93–101.
- Gillespie, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity. *Annual Review of Entomology* 42, 611–643.
- Hardin, J., Hilbe, J.M., 2003. *Generalized Estimating Equations*. Chapman & Hall, Boca Raton.
- Laloi, D., Sandoz, J.C., Picard-Nizou, A.L., Marchesi, A., Pouvreau, A., Tasei, J.N., Poppy, G., Pham-Delegue, M.H., 1999. Olfactory conditioning of the proboscis extension in bumble bees. *Entomologia Experimentalis Et Applicata* 90, 123–129.
- Maier, S.F., 2003. Bi-directional immune-brain communication: Implications for understanding stress, pain, and cognition. *Brain Behavior and Immunity* 17, 69–85.
- Maier, S.F., Watkins, L.R., 1998. Cytokines for psychologists: implications of bi-directional immune to brain communication for understanding behaviour, mood, and cognition. *Psychological Review* 105, 83–107.
- Mallon, E.B., Brockmann, A., Schmid-Hempel, P., 2003. Immune function inhibits memory formation in the honeybee *Apis mellifera*. *Proceedings of the Royal Society B* 270 (1532), 2471–2473.
- Mayntz, D., Raubenheimer, D., Salomon, M., Toft, S., Simpson, S.J., 2005. Nutrient-specific foraging in invertebrate predators. *Science* 307, 111–113.
- Moret, Y., Schmid-Hempel, P., 2000. Survival for immunity: The price of immune system activation for bumblebee workers. *Science* 290, 1166–1168.
- Mueller, U., 2000. prolonged activation of cAMP-dependent protein kinase during conditioning induces long-term memory in honeybees. *Neuron* 27, 159–168.
- Piomelli, D., 1994. Eicosanoids in synaptic transmission. *Critical Review of Neurobiology* 8, 65–83.
- Pugh, C.R., Fleshner, M., Watkins, L.R., Maier, S.F., Rudy, J.W., 2001. The immune system and memory consolidation: a role for the cytokine IL-1beta. *Neuroscience and Biobehavioral Reviews* 25, 29–41.
- Pugh, C.R., Nguyen, K.T., Gonyea, J.L., Fleshner, M., Watkins, L.R., Maier, S.F., Rudy, J.W., 1999. Role of interleukin-1 beta in impairment of contextual fear conditioning caused by social isolation. *Behavioural Brain Research* 106, 109–118.
- Smeets, P., Duchateau, M.J., 2003. Longevity of *Bombus terrestris* workers (Hymenoptera: Apidae) in relation to pollen availability, in the absence of foraging. *Apidologie* 34, 333–337.
- Stanley, D.W., 2000. *Eicosanoids in Invertebrate Signal Transduction*. Princeton University Press, Princeton.
- Stanley-Samuelson, D.W., Jensen, E., Nickerson, K.W., Tiebel, K., Ogg, C.L., Howard, R.W., 1991. Insect immune-response to bacterial-infection is mediated by eicosanoids. *Proceedings of the National Academy of Sciences of the United States of America* 88, 1064–1068.
- Wiesner, A., Wittwer, D., Gotz, P., 1996. A small phagocytosis stimulating factor is released by and acts on phagocytosing *Galleria mellonella* haemocytes in vitro. *Journal of Insect Physiology* 42, 829–835.