Rapid induction of immune density-dependent prophylaxis in adult social insects

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The innate immune system provides defence against parasites and pathogens. This defence comes at a cost, suggesting that immune function should exhibit plasticity in response to variation in environmental threats. Density-dependent prophylaxis (DDP) has been demonstrated mostly in phase-polyphenic insects, where larval group size determines levels of immune function in either adults or later larval instars. Social insects exhibit extreme sociality, but DDP has been suggested to be absent from these ecologically dominant taxa. Here we show that adult bumble-bee workers (*Bombus terrestris*) exhibit rapid plasticity in their immune function in response to social context. These results suggest that DDP does not depend upon larval conditions, and is likely to be a widespread and labile response to rapidly changing conditions in adult insect populations. This has obvious ramifications for experimental analysis of immune function in insects, and serious implications for our understanding of the epidemiology and impact of pathogens and parasites in spatially structured adult insect populations.

Keywords: density-dependent prophylaxis; innate immunity; sociality; *Bombus*

1. INTRODUCTION

The immune system is the evolutionary response to selective pressure from parasites and pathogens. Although vertebrates have both adaptive and innate immune responses, the vast majority of animal species rely upon the innate immune response. The innate immune system is costly to activate (Moret & Schmid-Hempel 2000) and costly to evolve (Kraaijeveld & Godfray 1997). Given these costs, particularly in the case of the former, it is unsurprising that the innate immune system exhibits plastic responses to perceived environmental threats, in the same way that phenotypic defences respond to environmental challenges

(Grant & Bayly 1981). A series of studies have demonstrated that phase-polyphenic insects respond to crowding, or group living, by upregulating their immune response, a phenomenon known as density-dependent prophylaxis (DDP) (Wilson & Reeson 1998; Wilson & Cotter 2008). This upregulation goes hand in hand with increased cuticular melanization (Reeson *et al.* 1998; Barnes & Siva Jothy 2000) and, in most cases, with enhanced defence against pathogens (Kunimi & Yamada 1990; Goulson & Cory 1995; Reeson *et al.* 1998; Wilson *et al.* 2002).

Despite the fact that they are the epitome of sociality, it has been suggested that DDP is absent from the eusocial insects (ants, some bees, some wasps and termites) (Pie *et al.* 2005). It has been suggested that this is due to the costs of DDP in permanently social organisms, and the presence of numerous other mechanisms that act to reduce infection (Pie *et al.* 2005). In addition, there is a mechanistic reason not to expect DDP in social insects. In other insects, DDP has been found to result from the detection of crowding by larvae, with DDP being expressed either in later instars or in adults, suggesting that the mechanism behind DDP is a developmental process. In contrast, the larvae of social insects, and particularly the bees and wasps, are unlikely to be able to detect colony density through larval crowding, as they are reared in individual compartments.

Here we investigate whether a plastic immune response, or DDP, can be elicited in adult social insects. Our results suggest that DDP may be a much broader adaptation than previously suggested, with clear implications for the understanding of epidemiology and disease dynamics in adult insect populations.

2. MATERIAL AND METHODS

Workers were taken from three colonies of *Bombus terrestris* (Koppert). Fifteen workers from each colony were randomly allocated to the *solitary* treatment, and 15 to the *social* treatment. All animals were transferred to plastic boxes (10.5 × 13 × 6 cm³) with fresh pollen and sugar water (50% v/v) (Apiinvert) ad libitum. All 90 bees were marked on the thorax with Tipp-Ex. Animals in the *solitary* treatment were kept in a box on their own, whereas animals in the *social* treatment were housed with an additional four workers from their own colony. Workers used in the experiment were checked for the presence of all known transmissible bumble-bee parasites (Schmid-Hempel 1998), and all workers had a clean bill of health. We did not control for bee age, due to the large number of workers required, but because workers were randomized across groups, age should not affect the experimental results.

After 8 days, each test individual was chilled on ice and the pleural membrane between the 5th and 6th sternite punctured with a sterile hypodermic needle. The droplet of haemolymph that came out of the wound was collected into a sterile, pre-chilled glass capillary. For each insect, 10 µl of haemolymph was collected and flushed into a 1.5-ml micro-centrifuge tube containing 50 µl of cold sodium cacodylate/CaCl₂ buffer (0.01 M sodium cacodylate, 0.005 M CaCl₂, pH 6.5). A 10-µl sub-sample was kept in a 0.5 ml micro-centrifuge tube coated with phenylthiourea and stored at –80°C until later examination for antibacterial activity using a zone-of-inhibition test. Phenylthiourea prevents melanin formation by inhibiting the activity of the phenoloxidase enzyme (Sugumaran *et al.* 1987). The methods for the zone-of-inhibition test were as described in Moret & Schmid-Hempel (2001). The remaining haemolymph solution was diluted with 30 µl of cold sodium cacodylate/CaCl₂ buffer and immediately stored at –80°C for later measurement of the prophenoloxidase system. For each individual, the haemolymph concentrations of the active phenoloxidase (PO) enzyme only and the proenzyme (proPO) in addition to that of the PO (total PO) were measured. PO was quantified without further

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Table 1. Results of the MANOVA analysis of immune function, with social context and colony as fixed factors. ZI, antibacterial activity; PO, phenoloxidase activity; PPO, total PO.

multivariate tests		Wilks' lambda	<i>F</i>	DF	<i>p</i>
social context		0.792	5.784	366	0.001
colony		0.824	2.235	6132	0.044
social context × colony		0.797	2.642	6132	0.019
univariate tests			<i>F</i>	DF	<i>p</i>
social context	ZI		4.624	168	0.035
	PO		12.769	168	0.001
	PPO		0.010	168	0.922
colony	ZI		0.056	268	0.056
	PO		0.065	268	0.065
	PPO		0.138	268	0.138
social context × colony	ZI		0.017	268	0.017
	PO		0.129	268	0.129
	PPO		0.031	268	0.031

activation, and total PO was assayed after activation of the proPO with chymotrypsin to produce active PO. For PO measurements, reaction mixtures contained 20 µl of haemolymph solution (dilution 1/20; haemolymph/sodium cacodylate/CaCl₂ buffer), 140 µl of distilled water, 20 µl of phosphate buffer saline (PBS: 8.74 g NaCl; 1.78 g Na₂HPO₄ · 2H₂O; 1000 ml distilled water; pH 6.5) and 20 µl of L-dopa solution (4 mg per millilitre of distilled water). For total PO measurements, the 140 µl of distilled water contained chymotrypsin (0.07 mg ml⁻¹) and the mixture was incubated for 5 min at room temperature before reading the enzymatic activity. The reaction was allowed to proceed at 30°C in a microplate reader (Versamax, Molecular Devices) for 40 min. Readings were taken every 10 s at 490 nm and analysed using SOFTmaxPRO 4.0 software (Molecular Devices). Enzyme activity was measured as the slope (*V*_{max} value) of the reaction curve during the linear phase of the reaction (Barnes & Siva Jothy 2000).

Data were analysed with a full-factorial MANOVA (table 1) with group (social versus solitary) and colony as fixed factors to examine differences in the following dependent variables: antimicrobial activity, measured as the diameter of the zone of inhibition in mm (ZI), PO, and total PO. Data fulfilled the assumptions of the test. Some animals died during the experiment, reducing sample size to a total of 34 solitary and 40 social animals. Data were analysed with SPSS16 for MacOS X.

3. RESULTS

There were significant interactions between colony and social context. Animals from colony 1 exhibited an increase in anti-bacterial activity and total PO in social situations, whereas animals from the remaining two colonies showed the opposite effect (ZI: $F_{2,68} = 4.329$, $p = 0.017$; total PO: $F_{2,68} = 3.672$, $p = 0.031$). However, there was no significant interaction effect for PO activity ($F_{2,68} = 2.114$, $p = 0.129$).

There were significant effects of group size on immune function (figure 1). Animals kept in social groups had 30 per cent higher PO activity ($F_{1,68} = 12.769$, $p = 0.001$; figure 1) and 36 per cent lower anti-bacterial activity ($F_{1,68} = 4.624$, $p = 0.035$; figure 1) than animals kept on their own. There was no effect of social context on total PO activity ($F_{1,68} = 0.010$, $p = 0.922$).

Figure 1. Animals in social groups had lower anti-bacterial activity (ZI) and higher phenoloxidase activity (PO). The figure shows the mean ± s.e. for the two branches of the immune system, open circles, ZI; filled circles, PO.

There were no effects of colony on any immune parameter (ZI: $F_{2,68} = 3.017$, $p = 0.056$; PO: $F_{2,68} = 2.850$, $p = 0.065$; total PO: $F_{2,68} = 2.037$, $p = 0.138$).

4. DISCUSSION

Social context elicits phenotypic plasticity in immune function in adult workers of the eusocial bumble-bee. Previous studies have demonstrated the impact of social context during larval development on either adult or late-instar immune function and resistance to parasites in insects (see §1). Our results suggest that such plasticity, or density-dependent prophylaxis, can also be elicited over very short timescales in adult insects. This significantly broadens the potential importance of DDP in understanding the dynamics of epidemiology and mortality in insect–pathogen systems.

Recent work suggests that the main frontline defence in insect immunity is the prophenoloxidase system, with anti-microbial peptides functioning to ‘mop up’ any remaining parasites or pathogens after the PO response (Haine *et al.* 2008). In our experiment, animals in social groups had significantly higher PO, indicating that groups are perceived as an increased pathogen/parasite threat and responded to by increasing frontline defences. This is in line with theories suggesting that sociality goes hand in hand with an increased disease threat (Alexander 1974). In addition to this increase in PO there was a decrease in antimicrobial peptides, providing further evidence for a physiological trade-off between these two branches of the immune system (Cotter *et al.* 2004), which may be due to competition for limiting protein resources (Povey *et al.* 2009).

An alternative explanation for our results might be that changes in immune function are a response to the stress of being in a group of a particular size (Steinhaus 1958). However, animals did not show general depression of their immune system either in the social or the solitary context (in each case, one branch of the immune system was upregulated and

the other downregulated), as would be expected if 'stress' were the cause (Reilly & Hajek 2008). Similarly, although workers in social groups compete for reproductive dominance (Honk *et al.* 1981), this is unlikely to lead to similar immune changes in dominant and subordinate animals (Sapolsky 2004), and because our samples were taken at random with respect to dominance from the social groups it is unlikely to be an explanation for our results.

Previous work suggested that DDP would be absent in social insects due to the plethora of alternative protective systems, and the fact that, by definition, social insects live in constant social conditions (Pie *et al.* 2005). However, both annual and perennial social insect societies go through significant population fluctuations. Our results suggest that, in such societies, adult animals can modulate their base immune function in an apparently adaptive way. Recent work found that immune function as measured by PO and haemocytes increased in bumble-bee workers as the colony aged (and increased in density) (Moret & Schmid-Hempel 2009). Our results provide a mechanism to explain this pattern.

To conclude, our results suggest that sociality may have selected for plasticity in the immune system, or DDP, in adult insects. This has implications both for the design of experimental studies of innate immunity, and for our understanding of the impact and epidemiology of parasites in the context of a socially variable host background.

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