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Testing male immunocompetence in two hymenopterans with different levels of social organization: 'live hard, die young?'

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Males are under different selective pressures than females, which results in differences in the physiology of the two sexes to maximize their fitness. In terms of immunity, males are typically considered as the 'sicker sex', where immunocompetence is reduced to favour increased reproductive output. However, male social Hymenoptera are also haploid and therefore lack allelic variation at the individual level, which can also lead to reduced immunocompetence. Over the last decade, several studies have provided evidence for a higher susceptibility to disease in males of social Hymenoptera, without clarifying whether this susceptibility was a direct consequence of their haploid condition or the result of a 'live hard, die young' overall evolutionary strategy. In the present study, we used an experimental approach of bacterial challenge to test the immune response of males and females in two species of social Hymenoptera (honey bees, *Apis mellifera*; paper wasps, *Polistes dominula*), where males show very different life-history traits. Drones benefit from colony protection for most of their life, whereas *P. dominula* males leave their colonies and have to survive for weeks at leks. If the haploid condition is responsible for a higher susceptibility in males, we should expect a lower immune response in males of both species compared to females. Conversely, if the immunocompetence depends on the life-history traits of males, an opposite trend is expected in males of the two species. Our results do not support the 'haploid susceptibility hypothesis' but are in accordance with the different life history of males from the two species. *Biological Journal of the Linnean Society*, 2014, ••, ••–••.

ADDITIONAL KEYWORDS: *Apis mellifera* – haploid susceptibility – immune challenge – *Polistes*.

INTRODUCTION

The wide range of differences between male and female phenotypes in nature is ultimately ascribable to ancestral asymmetry in the energetic cost of egg versus sperm production and in the parental effort. Under the evolutionary pressures of sexual selection, males are often bigger, stronger, and more aggressive relative to conspecific females. Most intriguingly, however, in terms of immunity, males usually represent the 'sicker sex' (Zuk, 2009). Over the years, many studies have demonstrated that parasites and pathogens may follow different patterns of infection depending on the host sex, with males being more vulnerable to diseases (Zuk, 1990). In arthropods, laboratory-based studies have confirmed an increased susceptibility of males (Schmid-Hempel, 2011) but, in the field, many environmental factors influence immunocompetence, and there is no clear demonstration of a universal and consistent sex bias (Sheridan *et al.*, 2000). There are competing hypotheses to explain the reduced immunocompetence shown by males in many species. First, there is a proposed

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trade-off between immunity and reproductive output, a sort of 'live hard, die young' hypothesis according to which males should direct their energies towards reproduction at the expense of immunity (Zuk, 2009). However, in haplodiploid species, such as social hymenoptera, there is an alternative hypothesis to explain the reduced immunocompetence of males. In these species, males are haploid and thus lack allelic variation in their genome compared to diploid females. This fundamental asymmetry has led to the 'haploid susceptibility hypothesis' (O'Donnell & Beshers, 2004): because of their haploidy, males are provided with less genetic variation to face new immune challenges and therefore they are more susceptible to diseases. Few empirical studies have tested this hypothesis in social hymenoptera (Baer *et al*., 2005; Baer & Schimd-Hempel, 2006; Gillespie, 2010; Laughton, Boots & Siva-Jothy, 2011; Retschnig *et al*., 2014), confirming a higher susceptibility of males under both field and laboratory conditions (but see Ruiz-González & Brown, 2006). In all these cases, however, it was not clear whether the higher susceptibility showed by males was a direct consequence of their haploid condition (i.e. reduction in the resistant alleles) or an adaptive life-history trait (Rolff, 2002): the result of a 'live hard, die young' overall evolutionary strategy in which males channel their resources towards mating behaviour and reproduction rather than towards immunity and survivorship (Zuk, 2009). To disentangle the question about which of the two hypothesis (i.e. 'live hard, die young' versus 'haploid susceptibility') is more likely to be operating in social Hymenoptera males, we compared the immune responses of male and female larvae of two species: the primitively eusocial wasp *Polistes dominula* and the highly eusocial honeybee *Apis mellifera*. Males of both species are haploid but exhibit considerable differences in their life-history traits. *Polistes dominula* males leave their nests a few days after eclosion and they establish, for several weeks, mating territories at landmarks, where they are exposed to a wide range of potential challenges, including pathogens and parasites (Beani, 1996). By contrast, *A. mellifera* drones follow a daily routine of leaving the colony to congregate in specific drone congregation areas where they look for a mate, and, if unsuccessful, they return to their colonies to recover their energies and be tended by workers (Ruttner, 1966; Koeniger, Koeniger & Pechhacker, 2005). Because drones leave the colony only for short periods, there is very little risk of diseases impacting upon their fitness compared to *Polistes* males: these have to survive for several weeks outside the protecting boundaries of their native nest. The present study aimed to test the haploid susceptibility hypothesis in these two species and to evaluate which aspect (genetics or life-history traits) has a major effect on immunocompetence.

MATERIAL AND METHODS

Insect collection and maintenance

Incipient colonies of the European paper wasp *P. dominula* were collected around State College, PA, in May 2012. Colonies ($N = 15$) were placed in 40 × 20 × 20 cm Plexiglas cages at room temperature and provided *ad libitum* with water, sugar, and wax moth larvae. Colonies were collected before worker emergence, when only one or few foundresses are on the nest. These were individually marked on the thorax with Testor's paint. Immediately after the emergence of the first workers, foundresses were permanently removed from six nests to allow newly-emerged workers to develop their ovaries and start laying unfertilized male eggs (Strassmann & Meyer, 1983). Ovarian development and egg laying in workers are experimentally triggered by removing the foundresess and all the eggs and the young larvae (Monnin *et al*., 2009). This procedure allows the production of male larvae early in the season because, typically, the production of sexuals (i.e. males and future queens) occurs only in late summer. Experimental infection of larvae from six queenless nests and nine queen-right colonies took place 5 weeks after foundress' removal, to ensure that all the brood present in the queenless nests were male larvae. At that time, the brood in queen-right colonies was composed of the first generations of female workers.

Colonies of *A. mellifera* ($N = 4$) were housed outside the Arthropod Research Facility at the Penn State University Park Campus (State College, PA). Honey bee colonies produce male and female larvae simultaneously in the spring and early summer, and thus no manipulation of the colonies was necessary. Because developmental stage may affect immune function (Wilson-Rich, Dres & Starks, 2008), we performed the experiment using immatures of comparable stages (fourth-instar larvae of both sexes).

For the immune challenge experiments (for more details, see below), larvae of both species were challenged outside the nest after being gently removed from their cells with forceps and placed individually into 24-well plates.

Bacterial infection with *Escherichia coli*

To test for the hypothesized reduction of individual immunocompetence in males, we injected larvae of both sexes and both species with bacteria and evaluated the bacterial clearance (i.e. numbers of bacterial cells eliminated) of male versus female larvae. We used the Gram-negative bacteria *E. coli* (strain NRRL

B-2422, mutant, streptomycin resistant), a common immune elicitor used to test immunocompetence in insects (Yang & Cox-Foster, 2005; Manfredini *et al*., 2010; Gätschenberger *et al*., 2013; Manfredini, Beani, & Grozinger, 2013). The pathogen is not naturally found in *P. dominula* or *A. mellifera* larvae. Therefore, we could exclude its presence in our larvae prior to artificial infection and the possibility of different host–pathogen coevolution patterns in the two systems. Bacterial cultures were grown overnight at 37 °C in Luria-Bertani (LB) broth containing Streptomycin at a concentration of 50 μg mL^{-1}. After centrifugation, bacteria were washed twice, resuspended in phosphate-buffered saline (PBS) and diluted to the desired concentration with PBS. The approximate amount of bacterial cells in the solution was determined using a haemocytometer (Neubar) and confirmed by plating the bacterial solution on LB agar and counting the colony forming units that grew overnight at 37 °C.

To infect paper wasp larvae, 1 μl of sterile PBS containing 1×10^4 bacterial cells was injected with a microsyringe (Hamilton) after cleaning the injection site with 90% ethanol. Subsequently, specimens were placed back in their plates and incubated for 24 h at 28 °C and 50% relative humidity. We used the same procedures on PBS-injected larvae and non-injected controls. After 24 h, samples were homogenized in sterile plastic tubes with beads (diameter 2 mm) using a Fast-Prep machine (MP Biomedicals) set at 45 s and 6 speed for one cycle. We serially diluted 10 μL of the homogenate and plated 100 μL of the ×1 and ×3 dilutions on sterile Petri Dishes containing LB agar and streptomycin (50 μg mL^{-1}). Plates were incubated at 37 °C for 24 h and the number of colony-forming units was recorded from each plate. We used a total of 45 male larvae (30 bacteria-injected, seven PBS-injected, eight controls) and 53 female larvae (34 bacteria-injected, nine PBS-injected, 10 controls), from six and nine colonies, respectively (at least five larvae per colony were used in our experiment). PBS-injected specimens and non-injected controls were handled in the same way as the experimental specimens.

The procedure used to challenge honey bee larvae and to evaluate their bacterial clearance was identical to that used for *Polistes* larvae with certain modifications: *A. mellifera* larvae were inoculated with a higher bacterial dose (1.5×10^5 bacterial cells) because of their larger size, and after injection, both drone (45 bacteria-injected, 19 PBS-injected, 20 controls) and worker (45 bacteria-injected, 17 PBS-injected, 21 controls) larvae (at least 20 larvae per colony were used) were incubated at 34 °C (instead of 28 °C) because this is the standard temperature of the brood nest of honey bee colonies.

The individual-level response of each larva to *E. coli* was processed after normalizing bacterial counts by $\log_{10}$ transformation. Because, even after transformation, the data showed evidence of non-normal distribution, differences in bacterial titres were analyzed by a nonparametric Mann–Whitney *U*-test.

RESULTS

Male and female wasp larvae exhibited significantly different response to *E. coli* infection (Fig. 1A). Male larvae had a significantly higher anti-bacterial response (or bacterial clearance) than female larvae because the bacterial loads found in their haemolymph were significantly lower than those in females (Mann–Whitney test: $Z = -2.043$, $P < 0.05$) (Fig. 1A). No bacteria were detected in the haemolymph of control or PBS-injected samples.

By contrast, *A. mellifera* female larvae showed significantly greater immunocompetence than male larvae (Mann–Whitney test: $Z = -2.457$, $P < 0.05$) (Fig. 1B). No bacteria were detected in the haemolymph of control or PBS-injected samples.

DISCUSSION

The present study demonstrates that phenotypic variation in immune response is affected by sex in social insects. However, the two species exhibited differential effects of sex, with higher immunocompetence (as predicted by the 'haploid susceptibility hypothesis'; O'Donnell & Beshers, 2004) in female *A. mellifera* relative to males, and higher immunocompetence in male *Polistes* wasps relative to females.

The reduced immunocompetence observed in honey bee drones is consistent with previous studies of social insects. Indeed, a previous study in honey bees, which analyzed phenoloxidase activity and antimicrobial peptides in different stages of drones and workers after an immune challenge with lipopolysaccharide derived from *E. coli*, demonstrated that drones had lower immunocompetence than workers in all developmental stages (Laughton *et al*., 2011). In the leaf-cutting ant *Acromyrmex echinatior*, there is a clear difference in immune response between males and workers, with the former exhibiting a much lower encapsulation response than the latter (Baer *et al*., 2005). A reduced encapsulation response was also found in male bumblebees *Bombus terrestris* with respect to workers (Baer & Schimd-Hempel, 2006; Gillespie, 2010). By contrast, higher immunocompetence in males (as exhibited by male larvae of *P. dominula* wasps) has been observed in at least another study on social insects. In *B. terrestris* challenged with *Crithidia bombi*, Ruiz-González & Brown

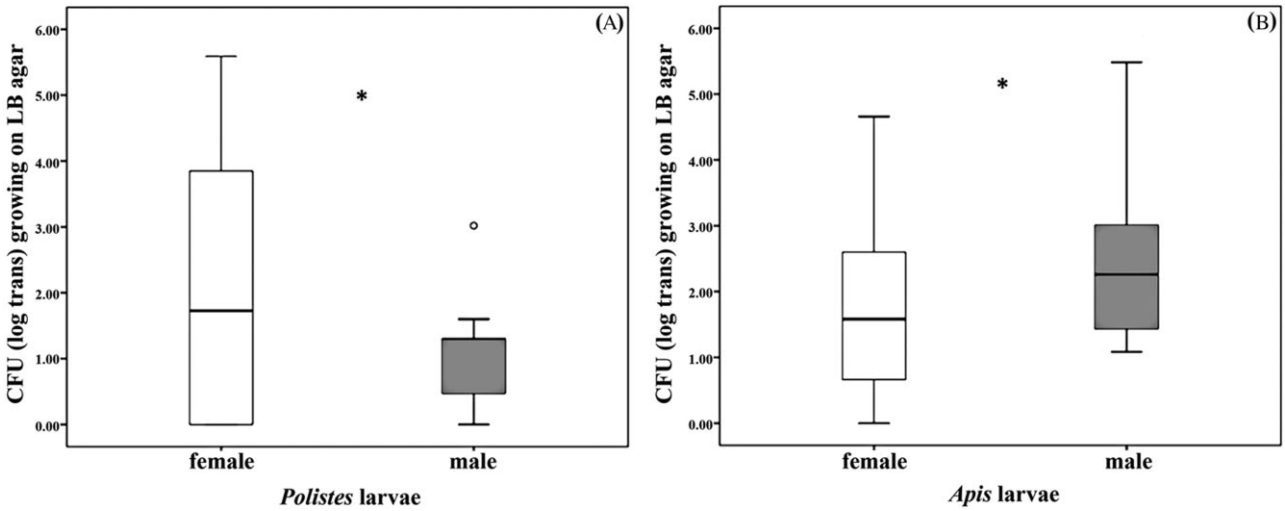

Figure 1. Larvae response to *Escherichia coli*. A, *Polistes* larvae. B, *Apis* larvae. The box plots represent the $\log_{10}$ transformation of the number of colony forming units (CFU) detected on LB agar plates from the haemolymph of male and female larvae after 24 h of incubation at 37 °C. Box plots show the median, 25–75% percentiles, range, and outliers (*$P < 0.05$).

(2006) found no difference in either the initial susceptibility or intensity of infection between haploid males and diploid workers. It was suggested that this parasite may simply be more adapted to the common female host, and thus male haploid susceptibility might be hidden by parasite adaptation to achieve higher infection levels in females. In *P. dominula*, the parasitic castrator *Xenos vesparum* dramatically alters female hosts, by inhibiting ovary activation and causing them desert the colony (but not male hosts) in terms of both sexual performance and reproductive apparatus (Cappa *et al*., 2014).

The species differences in the effects of sex on immunocompetence may be a result of differences in male life histories: sexual dimorphism in immune systems may be the result of sex-divergent selective pressures. Drones of *A. mellifera* are 'embedded in the protective social network and leave the colony only for short, synchronized mating flights' (Streinzer *et al*., 2013). A colony-protected male has very little risk of diseases and, consequently, is predicted to invest less in immune defences. By contrast, *P. dominula* males, similarly to some bumble bees (Goulson, 2003), spend several weeks away from their colonies. Males form leks in July, whereas reproductive gynes (i.e. future queens) visit leks to mate usually in September, when only persistent males are still present. Thus, males that can resist pathogens, parasites, and other stressors have the greatest reproductive success (the 'marathoner hypothesis', Beani, 1996).

In conclusion, the results of the present study, reporting a difference between the two species in the developmental stage where it was less likely to be found, suggest that not only genetic condition, but also an ensemble of complex factors and trade-offs related to male life history can affect male immune responses in social Hymenoptera. Clearly, further comparative studies across species are needed to investigate why males of different species vary in their immune responses at the cellular and humoural levels, in response to parasites or pathogens, as well as to verify the exact roles of the haploid condition versus life-history traits (which are shaped by the social organization of colonies in social insects) with respect to determining the level of susceptibility.

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