Insectes Sociaux (2019) 66:91–105
https://doi.org/10.1007/s00040-018-0668-x

Insectes Sociaux

RESEARCH ARTICLE

# An improved method for testing invertebrate encapsulation response as shown in the honey bee

N. Wilson-Rich[1,2] · R. E. Bonoan[1] · E. Taylor[1] · L. Lwanga[1] · P. T. Starks[1]

Received: 26 February 2018 / Revised: 26 September 2018 / Accepted: 6 October 2018 / Published online: 25 October 2018


## Abstract

In 1882, Metchnikoff documented the encapsulation response (ER) of the invertebrate immune system. Since then, researchers have used Metchnikoff's method to quantify immune function—and examine its relationship with ecological and behavioral factors—across various insect taxa. While scientists continue to uncover information regarding invertebrate immunity, behavioral ecology, and ecological immunology, the basics of Metchnikoff's method have remained unchanged. All but two previous studies investigating insect immunity have used sterile or PBS-coated inducers, although we know that the immune system recognizes specific pathogens. To account for the specificity of the immune system, we modified Metchnikoff's method and coated nylon monofilaments with pathogen-associated molecular patterns (PAMPs). Using honey bees (*Apis mellifera*), we examined ER using implants coated with PAMPs ("PAMPlants") found on known honey bee parasites and pathogens. Lipopolysaccharide (LPS), peptidoglycan (PGN), and β-1, 3-glucan (B13G) PAMPlants mimicked an infection with Gram-negative bacteria, Gram-positive bacteria, and fungi, respectively. Our PAMPlants induced stronger responses than the control implants in both singly- (one PBS-coated or PAMP-coated implant) and doubly- (internal control; one PBS-coated and one PAMP-coated implant) implanted animals. In doubly-implanted individuals, there was a significant increase in response to B13G and LPS when compared with internal controls. The PGN and BSA did not differ from the internal controls in the doubly implanted individuals. These methods provide an improvement when exploring responses to specific pathogens and exploring topics within the field of invertebrate ecological immunity. When applied to social systems, these methods can be used to examine the evolution of disease resistance in societies.

**Keywords** Disease resistance · Innate immunity · Insect societies · Invertebrate immunity · Sociality

## Introduction

A common cost of social living is the spread of disease (Wilson 1975). Many individuals living in close association lead to frequent physical interaction and thus many opportunities for disease to spread. Furthermore, many related individuals in one place lead to lower genetic diversity which can facilitate the spread of disease. Despite these threats, insect societies are wildly successful (Wilson 1971). For many insect societies, the first line of defense against invaders is behavioral. For example, when a honey bee colony is infected with a heat-sensitive pathogen, workers collectively raise the temperature of the hive to generate a preventative colony-level fever (Starks et al. 2000).

One hypothesis that may explain the success of insect societies in spite of disease threats is the relaxed selection hypothesis, which postulates that social species have evolved behavioral immune responses, such as honey bee fever, that decrease both disease risk to the group as well as the need for strong individual immunity. When behavioral defenses are not successful, however, individuals must turn to physiological defenses. The social group hypothesis postulates that due to the increased risk of disease spread in societies,

Noah Wilson-Rich and Rachael E. Bonoan contributed equally.

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s00040-018-0668-x) contains supplementary material, which is available to authorized users.

✉ P. T. Starks
philip.starks@tufts.edu

[1] Department of Biology, Tufts University, Medford, MA, USA

[2] Present Address: Urban Beekeeping Laboratory and Bee Sanctuary, Inc., Best Bees Company, Llc, 839 Albany Street, Boston, Ma 02119, USA

sociality may lead to stronger physiological immunity in individuals. In support of the relaxed selection hypothesis, Lopez-Uribe et al. (2016) have shown reduced physiological response to lipopolysaccharide, a protein on Gram-negative pathogens, in social insect lineages. Here, we describe a method to investigate physiological immunity, and tease apart the two hypotheses, in response to various classes of pathogens.

Like vertebrates, an invertebrate's first line of defense to a physiological assault is a physical barrier (Antunez et al. 2009). For insects, the exoskeleton's cuticle and the digestive tract's peritrophic membranes serve as protection. When an infection evades such barriers, the host must recognize the invader as both foreign and dangerous to launch the appropriate immune response. In invertebrates, this process is carried out via proteins called pattern recognition receptors (PRRs), and other recognition molecules, that recognize specific, highly conserved pathogen-associated molecular patterns (PAMPs) found on the microbial cell wall (reviewed in Nurnberger et al. (2004); Zipfel and Felix (2005)). For example, peptidoglyan (PGN) is an essential component of the bacterial cell wall in both Gram-positive and Gram-negative bacteria and thus serves as a PAMP recognized by the invertebrate immune system. In addition to PGN, the cell wall of Gram-negative bacteria also contains the endotoxin lipopolysaccharide (LPS) that is recognized by host PRRs. PAMPs such as β-1, 3-glucans (B13G), mannoproteins, and phospholipomannan are found on fungi.

Upon recognition of a foreign body, insects can employ one or more of three general innate responses: (1) synthesis of antimicrobial peptides; (2) initiation of proteolytic cascades, which lead to clotting and melanization; (3) phagocytosis or encapsulation by hemocytes (Kimbrell and Beutler 2001). For the purposes of this study, we focused on the encapsulation response (ER) by which the host immune system surrounds a foreign body with multiple hemocytes. The bound hemocytes then isolate and neutralize foreign bodies via anoxia, toxic reactive oxygen species, or starvation (reviewed in Moreno-Garcia et al. (2013)). In invertebrates, the encapsulation response is highly effective at clearing small foreign bodies or bacteria (Smith 2016). The first observation of the ER, and the creation of the assay to measure ER, is attributed to Élie Metchnikoff (Beck and Habicht 1996; Chernyak and Tauber 1988; Gordon 2008; Tauber 2003).

Although he began as a zoologist and embryologist, Metchnikoff is now referred to as "The Father of Natural Immunity" (Beck and Habicht 1996; Gordon 2008). In 1908, Metchnikoff won the Nobel Prize in medicine for research stemming from his novel methods of measuring immune strength in invertebrates (Metchnikoff 1884, as translated and reprinted in Beck and Habicht (1996) and references therein). While vacationing with his family on the Sicilian coast in 1882, Metchnikoff implanted a rose thorn into a starfish larva, observed an ER, and thus invented the first method of studying the invertebrate ER (Beck and Habicht 1996; Tauber 2003).

Nearly a century later, Metchnikoff's method for measuring invertebrate immune function was resurrected and used to answer questions relating to ecological immunity—the study of how abiotic and biotic factors influence variation in immune traits. As the immune system of invertebrates is simpler than that of vertebrates, and is phylogenetically conserved, an understanding of invertebrate ecological immunity provides insights in the evolution of immunity overall (Beck and Habicht 1996). Recent empirical ER studies (Table 1) using Metchnikoffian methods of Konig and Schmid-Hempel (1995) have elucidated relationships between immune function and many ecological and behavioral factors in insects, including: sex, behavioral role, dominance, female mate choice, foraging ability, diet, habitat type, infection risk, co-infection, energy cost, individual and colony condition, ontogeny, and invasion biology (Table 1 and references therein). In addition, as exhibited by Lopez-Uribe et al. (2016), studying ecological immunity in insect societies provides insight into the evolution of sociality and how individuals deal with the spread of disease in close quarters.

In this paper, we report results from an improved approach to the classic method for testing immune function using foreign bodies coated with PAMPs. All but two prior insect ER studies (Table 1) employed implants (monofilaments or beads) that were either sterile and uncoated or sterile and coated with PBS. The two studies (Appler et al. 2015; Lopez-Uribe et al. 2016) that used PAMP-coated implants only used one type of PAMP: LPS. Our comparative method allows for further investigation of the ability of a host to identify and respond to a specific class of pathogen. Furthermore, of the studies listed in Table 1, only 15 (about 20%) focus on a social study system. Investigating physiological immunity in response to various classes of pathogens in social insects could distinguish between, or support both, the social group hypothesis and the relaxed selection hypothesis. It is possible, for example, that while the relaxed selection hypothesis holds true for Gram-negative pathogens (see Lopez-Uribe et al. (2016)), the social group hypothesis holds true for Gram-positive and/or fungal pathogens.

Since much is known concerning both behavioral and physiological immune mechanisms of honey bees (*Apis mellifera*), this social insect served as our study system (Wilson-Rich et al. 2008; Wilson-Rich et al. 2014; Wilson-Rich et al. 2009). Aside from immune mechanisms—including ER—being well documented, further understanding immunity in the honey bee is of ecological and economic importance. As the honey bee is naturally challenged by various pathogens and parasites, and its genome is fully sequenced (Honeybee

**Table 1** Findings uncovered using various Metchnikoffian methods with different insect study systems

| | Study system | Method used | Major findings | References |
|---|---|---|---|---|
| 1 | Insects of varying sociality (bees, ants, wasps, termites) | LPS-coated monofilament | Within social insects, there is a negative effect of colony size on ER | Lopez-Uribe et al. (2016) |
| *Eusocial insects* | | | | |
| 2 | *Apis mellifera* (honey bee) | LPS-coated monofilament | More variation in ER for bees living in rural landscapes than in urban landscapes; no difference in ER between feral and managed bees | Appler et al. (2015) |
| 3 | | Uncoated monofilament | No change in ER across larvae, pupae, young adults (nurses), and old adults (foragers) | Wilson-Rich et al. (2008) |
| 4 | | Uncoated monofilament | Challenging larvae with nylon implants enhanced nitric oxide production and the spread of hemocytes | Negri et al. (2014) |
| 5 | | Uncoated monofilament | ER of bees treated with neonicotinoids is significantly reduced compared to control bees | Brandt et al. (2016) |
| 6 | *Bombus terresteris* (bumble bee) | Uncoated monofilament | Workers allowed to forage had lower ER than those prevented from foraging; strong colony effect on ER | Konig and Schmid-Hempel (1995) |
| 7 | | Uncoated monofilament | ER was not affected by diet or body mass | Schmid-Hempel and Schmid-Hempel (1998) |
| 8 | | Uncoated monofilament | Males had lower ER compared to sister workers; ER was lower in late-produced and late-reproducing cohorts compared to early cohorts | Baer and Schmid-Hempel (2011) |
| 9 | | Uncoated monofilament of 1 mm or 2 mm length | ER was not affected by the size of implant or by *C. bombi* co-infection | Allander and Schmid-Hempel (2000) |
| 10 | *Formica exsecta* (wood ant) | Uncoated monofilament | Males have lower ER than queens; among males, micraners had lower ER than macraners and males from larger nests had stronger ER; among queens, no relationship between ER and body size or colony | Vainio et al. (2004) |
| 11 | *Formica aquilonia* (forest dwelling ant) | Uncoated monofilament rubbed with sandpaper | ER was similar between ants in heavy metal environments and translocated groups; ER was elevated in moderate, whereas suppressed in high heavy metal levels suggesting higher risk for infections in heavily polluted areas | Sorvari et al. (2007) |
| 12 | *Zootermopsis angusticollis* (dampwood termite) | Uncoated monofilament | ER did not differ between inbred and outbred termites | Calleri et al. (2006) |
| 13 | | Uncoated monofilament | Sex, implant period, and colony of origin significantly influenced ER, however, mass and mated/unmated status did not affect ER | Calleri et al. (2007) |

**Table 1** (continued)

| | Study system | Method used | Major findings | References |
|---|---|---|---|---|
| *Primitively eusocial insects* | | | | |
| 14 | *Polistes dominulus, P. fuscatus* (paper wasps) | Uncoated monofilament | The invasive *P. dominulus* a had lower ER than the native, sympatric, congener, *P. fuscatus* | Wilson-Rich and Starks (2010) |
| 15 | | Uncoated monofilament | Late season workers exhibit lower ER than early season workers | Wilson-Rich et al. (2014) |
| *Non-eusocial insects* | | | | |
| 16 | *Acheta domesticus* (house cricket) | Uncoated monofilament | Supports immunocompetence handicap hypothesis by showing male crickets with more song syllables (preferred by females); also had higher ER | Ryder and Siva-Jothy (2000) |
| 17 | *Aedes aegypti* (yellow fever mosquito) | Injected, uncoated beads | Mosquitoes injected with malaria parasites had reduced ER versus control injections | Boete et al. (2002) |
| 18 | *Aglaoctenus lagotis* (spider, no common name) | Uncoated monofilament | Females constructing webs had higher ER than those prevented from constructing webs; males had lower ER than females | González et al. (2015) |
| 19 | *Anticarsia gemmatalis* (velvetbean caterpillar) | Uncoated monofilament | ER was not affected by development temperature or population density, however, caterpillars of the intermediate color phenotype showed greatest ER | Silva and Elliot (2016) |
| 20 | *Anabrus simplex* (Mormon cricket) | Uncoated glass rod | ER directly proportional to body mass, but unaffected by the dietary treatments in the short term | Srygley et al. (2009) |
| 21 | | Uncoated glass rod | ER directly correlated with body mass, crickets fed a carbohydrate diet had significantly greater ER than those fed proteins, males had slightly greater encapsulation response than females | Srygley and Lorch (2011) |
| 22 | | Uncoated glass rod | Individuals fed low protein diets exhibited a slower ER than those fed high protein diets | Srygley and Jaronski (2018) |
| 23 | *Calopteryx splendens* (damselfly) | Uncoated monofilament | ER positively correlated with both male wingspots size and hemocyte number, but negatively correlated with wingspot asymmetry | Rantala et al. (2000) |
| 24 | | Uncoated monofilament | No relationship between ER and wing pigment heterogeneity; no effect of pre- or post-implantation PO activity levels in males | Siva-Jothy (2000) |
| 25 | | Uncoated monofilament | No effect of copulatory activity on ER | Krams et al. (2015b) |

**Table 1** (continued)

| | Study system | Method used | Major findings | References |
|---|---|---|---|---|
| 26 | *Calopteryx virgo* (damselfly) | Uncoated monofilament | Males winning male–male fights had higher ER and higher post-fight fat reserves than losing males | Koskimaki et al. (2004) |
| 27 | *Camponotus fellah* (ant, no common name) | Uncoated monofilament and injection with PGN | Workers injected with PGN had reduced ER compared to ringer injection; workers did not decrease the level of self-grooming or allogrooming | de Souza et al. (2008) |
| 28 | *Cerastoderma edule* (edible cockle) | Injected, uncoated beads and uncoated monofilament | Positively charged targets stimulated the most vigorous ER; non-specific electrostatic forces and humoral plasma factors have a synergistic role on ER | Wootton et al. (2006) |
| 29 | *Coenagrion puella* (azure damselfly) | Uncoated monofilament | Larvae exposed to UV exhibited higher ER and metamorphosed later than control larvae | Debecker et al. (2015) |
| 30 | *Diaphania hyalinata* (Melonworm) | Uncoated monofilament | ER negatively affected by superparasitism by the parasitoid *Palmistichus elaisis* | Pereira et al. (2017) |
| 31 | *Deinacrida rugosa* (Cook Strait giant weta) | Uncoated monofilament | On average, males have higher ER than females; fed individuals tend to have higher ER than starved individuals | Kelly (2016) |
| 32 | *Epirrita autumnata* (autumnal moth) | Uncoated monofilament rubbed with sandpaper | ER is positively correlated with plant defenses during a moth population increase | Kapari et al. (2006) |
| 33 | | Uncoated monofilament rubbed with sandpaper | Several individual hydrolysable tannins were negatively associated with ER; flavenoid glycosides had no relationship with ER | Haviola et al. (2007) |
| 34 | | Uncoated monofilament rubbed with sandpaper | Larvae reared on low-quality food had higher ER as pupae than larvae reared on high-quality food; females had higher ER than males | Klemola et al. (2007) |
| 35 | *Euoniticellus intermedius* (dung beetle) | Uncoated monofilament | ER positively correlated with both age and elytra length; no difference between sexes | Pomfret and Knell (2006) |
| 36 | | Uncoated monofilament rubbed with sandpaper | Gynes had stronger ER than workers; habitat type affected ER differently between castes | Sorvari et al. (2008) |
| 37 | *Galleria mellonella* (greater wax moth) | Injected, Congo red-stained beads | Priming with heat-killed bacteria causes dose-dependent elevation in ER | Wu et al. (2014) |
| 38 | | Uncoated monofilament | ER response was stronger in larvae raised on food of average nutritional quality when compared with larvae raised on a high-energy diet | Krams et al. (2015a) |
| 39 | | Uncoated monofilament | Larval diet and gut microbiome affect ER | Krams et al. (2017) |

**Table 1** (continued)

| | Study system | Method used | Major findings | References |
|---|---|---|---|---|
| 40 | *Gryllus bimaculatus* (field cricket) | Uncoated monofilament | Dominant males obtained more mates and had higher ER than size-matched subordinates; no correlation between weight and ER | Rantala and Kortet (2004) |
| 41 | | Uncoated monofilament | In both sexes, ER was negatively correlated with body size, development time and lytic activity | Rantala and Roff (2005) |
| 42 | | Uncoated monofilament rubbed with sandpaper | Female crickets preferred courtship songs from males with a high ER; ER did not correlate with male body weight | Rantala and Kortet (2003) |
| 43 | *Gryllus firmus* (field cricket) | Injected, uncoated beads | Long-winged morphs had a higher ER than short-winged morphs | Kirschman et al. (2017) |
| 44 | *Gryllus vocalis* (field cricket) | Uncoated monofilament rubbed with sandpaper | Following experimentally assigned matings, females showed higher ER than males; the number of matings (5 versus 10) had no effect on ER | Gershman (2008) |
| 45 | Helicoverpa armigera (cotton bollworm) | Injected, uncoated beads | HaCTL3, a C-type lectin, enhances ER | Wang et al. (2017) |
| 46 | | Injected, uncoated beads | Fungal infection reduced ER | Zhong et al. (2017) |
| 47 | | Injected, uncoated beads and PBS | bead challenge stimulated release of growth blocking peptide genes HaGBPB1 and HaGBPB2 into plasma | Zhuo et al. (2018) |
| 48 | | In vitro assay with PBS-coated beads | Immune-related gene Ha-DFP1 is involved in ER | Hu et al. (2017) |
| 49 | *Hemideina crassidens* (tree weta) | Uncoated monofilament | Males exhibited significantly greater ER than females | Kelly and Jennions (2009) |
| 50 | | Uncoated monofilament | Mated females showed an increase in ER | Kelly (2017) |
| 51 | *Hemideina maori* (mountain stone weta) | Injected, uncoated beads | The relationship between beads recovered and the number of beads melanized was positive for all four morphs by sex combinations | Robb et al. (2003) |
| 52 | *Hetaerina americana* (American rubyspot damselfly) | Uncoated monofilament | Territorial males had higher levels of pigmentation, while fat reserves and immune defense were constant during territorial tenure | Contreras-Garduno et al. (2006) |
| 53 | *Hygrolycosa rubrofasciata* (wolf spider) | Uncoated monofilament | ER was higher among males with higher drumming rates, but not affected by mobility or body mass | Ahtiainen et al. (2004) |
| 54 | *Lepidopteran* spp. (caterpillars) | Injected, uncoated beads | Negative correlation between ER and parasitism incidence | Smilanich et al. (2009) |

**Table 1** (continued)

| | Study system | Method used | Major findings | References |
|---|---|---|---|---|
| 55 | | Injected, PBS-coated beads | Within 24 h of inoculation, parasitized larvae exhibited a lower ER than unparasitized larvae | Teng et al. (2016) |
| 56 | *Leptinotarsa decemlineata* (Colorado potato beetle) | Uncoated monofilament | Primary inoculation with fungus did not affect ER in larvae, primary inoculation with bacteria decreased ER in larvae | Yaroslavtseva et al. (2017) |
| 57 | *Leucorrhinia intacta* (dot-tailed whiteface dragonfly) | Uncoated monofilament | Larvae exposed to caged predators had a higher ER than those not exposed to caged predators | Duong and McCauley (2016) |
| 58 | *Paropsis atomaria* (common leaf beetle) | Uncoated monofilament | Larvae raised on plants under elevated $CO_2$ exhibited a higher ER than those raised on control plants | Gherlenda et al. (2016) |
| 59 | *Pieris brassicae* (white cabbage butterfly) | Uncoated monofilament | Implanted individuals increased their standard metabolic rate by approximately 8% compared to controls, hence an energetic cost to ER | Freitak et al. (2003) |
| 60 | *Protophormia terraenovae* (blow fly) | Uncoated monofilament | Temperature influenced sex differences in immune defense; males had a stronger ER at higher temperatures when compared with females | Polkki et al. (2014) |
| 61 | Schistocerca gregaria (desert locust) | Uncoated monofilament | No difference in ER between social and solitary phases of living | Wilson et al. (2002) |
| 62 | *Schizocosa ocreata* (brush-legged wolf spider) | Uncoated monofilament | Significant positive correlation between tuft size (ornamentation) and ER; males that performed 5 min of courtship had lower ER than those that did not perform courtship | Gilbert and Uetz (2016) |
| 63 | *Spodoptera littoralis* (cotton leafworm caterpillar) | Uncoated monofilament | ER was great in individuals on a high protein-to-carbohydrate ratio diet than those on a low ratio diet | Lee et al. (2006) |
| 64 | | Injected, PBS-coated beads | ER depends on an immune gene, 102 SI | Di Lelio et al. (2014) |
| 65 | *Tenebrio molitor* (mealworm beetle) | Uncoated monofilament | Individuals treated with juvenile hormone (JH) treatment group exhibited lower ER and PO activity than the non-JH treated control group | Rantala et al. (2003) |
| 66 | | Uncoated monofilament | No difference in the survival curves of males implanted with one, two, or no implants when the males were starved; males lost less body mass when immune challenged and given an opportunity to feed | Vainikka et al. (2007) |
| 67 | | Uncoated monofilament | ER of males in the single-treatment group exhibited significantly greater ER than that of the repeat-treatment males | Kivleniece et al. (2010) |

**Table 1** (continued)

| | Study system | Method used | Major findings | References |
|---|---|---|---|---|
| 68 | | Uncoated monofilament | Inbreeding did not significantly affect ER; however, it did significantly reduce resistance against *Beauveria bassiana* fungi | Rantala et al. (2011) |
| 69 | | Uncoated monofilament | Significant increase in ER between the first and second implantations in males | Daukste et al. (2012) |
| 70 | | Uncoated monofilament | Survival of beetles subjected to immune activation by a nylon implant previous to fungal exposure was higher than survival of beetles which were subjected to fungal infection only | Krams et al. (2013) |
| 71 | | Uncoated monofilament | ER and development time decreased with a rise in temperature | Prokkola et al. (2013) |
| 72 | | Uncoated monofilament and monofilament attached to a Malpighian tubule tissue graft | ER results in significant damage to Malpighian tubule; physiology in the proximate area to the nylon implant | Sadd and Siva-Jothy (2006) |
| 73 | | Uncoated monofilament rubbed with sandpaper | Females were more attracted to pheromones from males with high ER; ER correlated with PO enzyme activity and fresh body weight | Rantala et al. (2002) |
| 74 | | Uncoated monofilament rubbed with sandpaper | Individuals with a darker cuticle exhibited a higher ER | Kangassalo et al. (2016); Krams et al. (2016) |
| 75 | | In vitro assay with uncoated beads and ectoparasitoid venom | Ectoparasitoid venom inhibits ER of host hemocytes, but not in a dose-dependent manner | Li et al. (2018) |
| 76 | Tetrix undulate (pygmy grasshopper) | Uncoated monofilament | No dependence of ER on individual body size | Civantos et al. (2005) |

Appler et al. and Lopez-Uribe et al. to be highlighted in gray, used sterile and/or PBS-coated implants. Only 15 of the 76 studies examine ER in social insects

Genome Sequencing Consortium 2006), immune function can be studied under near-natural conditions and linked to specific immune genes for phylogenetic analysis (e.g. Lopez-Uribe et al. (2016)).

While honey bees are affected by many pests and pathogens, the most notorious is the parasitic mite, *Varroa destructor*. In adult honey bees, the *Varroa* mite implants itself into the host cuticle (Martin 2001; Spivak 1996). Even if the mite itself does not induce ER, it creates a structural insult for other pathogens and parasites to pass through, and is the main cause of disease spread in a hive (Kang et al. 2016). Honey bee colonies affected by large numbers of mites suffer from Parasitic Mite Syndrome, which causes the population to dwindle and the colony to eventually die out (Sammataro et al. 2000). Our PAMPplants mimic this natural process while focusing on the immune response of the host to the bacterial and fungal pathogens commonly transmitted by *Varroa* (Mariani et al. 2012; Vanikova et al. 2015). We did this using three different PAMPs to coat the implants: PGN, LPS, and B13G (reviewed in Sammataro et al. (2000)). Due to the historical relationship between pathogens and host immune system (van Engelsdorp and Meixner 2010), each PAMP coating was predicted to elicit a more specific ER relative to control implants.

Each study listed in Table 1 added valuable information to the field of insect immunity. By incorporating pathogen-specific molecules on experimental implants, we hope to better mimic the natural host–pathogen relationship. In addition, in doing so, provide an improved method to researchers to create a clear picture of ecological immunity in insects and invertebrates in general. Applied across various levels of sociality, our method could be used to better understand the interplay of the social group hypothesis and the relaxed selection hypothesis in the evolution of disease resistance and sociality in insect societies.

## Materials and methods

To create the PAMPlants, sterile (rinsed with 70% ethanol) cuts of nylon monofilament (2 mm long, 0.4 mm diameter, Scientific Anglers Tippet, 3M, diameter previously incorrectly reported in Wilson-Rich et al. (2008)) were dipped through one of the five different solutions with varying concentrations: (1) phosphate-buffered saline (PBS, our control); (2) lipopolysaccharide in PBS (LPS; 0.1 mg/ml, 1 mg/ml, and 10 mg/ml); (3) peptidoglycan in PBS (PGN; 1 mg/ml, 10 mg/ml, and 100 mg/ml); (4) β-1,3-glucans in PBS (β13G; 1 mg/ml, 10 mg/ml, and 100 mg/ml); and (5) bovine serum albumin in PBS (BSA; 1 mg/ml, another control). As such, there was a proportional fold increased in PAMP concentrations across treatments. PBS and BSA monofilaments were controls, and expected to induce a baseline ER. Implants were allowed to dry overnight in sterile petri dishes.

To quantify the ER of individuals to the PAMPlants, we performed a series of modified, standard ER assays (Konig and Schmid-Hempel 1995; Wilson-Rich et al. 2008) over 2 years (2008, 2009). Each year, we collected foraging honey bees—which are all similar age (Winston 1987)—at the entrance of a single hive maintained at Tufts University in Medford, MA. This allowed us to control for genetic variation and health status. Individuals were stored collectively and immediately brought to the laboratory for experimentation.

Collected foragers were ice-anesthetized and the nylon monofilaments were implanted between the third and fourth ventral intersegmental membrane ($N=74$ singly implanted, and $N=102$ doubly implanted, individuals). All implants remained in situ for 4 h [see Wilson-Rich et al. (2008) and references therein]. Experimental individuals in 2008 received a single implant, either an experimental PAMPlant or a control implant coated with PBS. Experimental individuals in 2009 received double implants, with one PAMPlant matched with an internal PSB-coated control implant for a self-paired design. To control for side, side was switched for PAMPlant and control implants, and the left side was always inserted first, thus controlling for which condition (PAMPlant or control implant) was performed first. In comparison with PBS, an additional control—an insert coated with BSA—was used, because unlike LPS, PGN, and β13G, it is predicted to elicit a baseline immune response.

After 4 h, experimental individuals were re-anesthetized on ice and the implants were removed with fine forceps. The removed implants were then analyzed for mean gray value using a fluorescence-detecting microscope (Olympus VX40), and image capturing (Optronics Magna Fire-SP, v1.0_5) and analysis [ImageJ (NIH)] software [see Wilson-Rich et al. (2008) and references therein]. Darker implants indicated more melanization, which served as a proxy for a stronger ER (Appler et al. 2015; Lopez-Uribe et al. 2016; Wilson-Rich et al. 2008), and thus greater immune function according to this measure, see Online Appendix 1 for a detailed description of the methods.

### Statistical methods

Singly implanted mean ER values were compared using ANOVA followed by post-hoc Tukey HSD pairwise comparisons. Doubly implanted mean values within PAMP treatments (i.e., between PAMP-coated and PBS-coated implants or PBS-coated and BSA-coated implants) were compared using paired *t* tests within individuals. The ER to different PAMPlants in doubly implanted individuals was compared using ANOVA followed by post-hoc Tukey HSD pairwise

comparisons. All statistics were calculated using SPSS for Windows v. 11.0.

## Results

There were no significant effects of PAMP concentration on ER (Two-way ANOVA, $F = 1.12$, $df = 3$, $p = 0.35$) (Supp. Fig. 1). As such, data from all samples were combined within the singly- and doubly implanted groups. Overall, the PAMPlants induced higher ERs than the control (PBS, BSA) implants (Figs. 1, 2).

In 2008, honey bees with a single implant exhibited a significantly upregulated ER in response to each of the three PAMPs when compared with the control (PBS-coated) implants (One-way ANOVA, $F = 8.71$, $df = 3.70$, $p < 0.001$). There were no significant differences in ER across the three PAMP treatments (Fig. 1).

In 2009, within doubly implanted individuals, PAMPlants induced stronger responses than PBS-coated implants. Compared with the PBS-coated control, this relationship was significant for two PAMPs: B13G (paired $t$ test, $t = 3.30$, $df = 22$, $p = 0.02$), and LPS (paired $t$ test, $t = 1.85$, $df = 25$, $p = 0.04$) (Fig. 2). PGN and BSA showed no overall difference from the internal PBS control. There was no effect of side (left versus right) on ER. Across all treatments, doubly implanted individuals had the strongest ER to B13G (Tukey's HSD, $p_{B13G-LPS} = 0.03$, $p_{B13G-PGN} < 0.001$, $p_{B13G-BSA} < 0.001$). There was no difference in ER between LPS, PGN, or BSA within doubly implanted treatments.

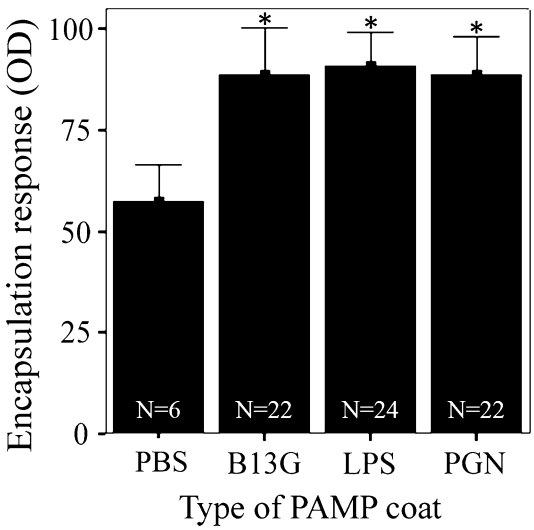


**Fig. 1** Mean encapsulation response (as measured via optical density, OD) of singly implanted honey bees (*Apis mellifera*) ± 1 SE. Adult bees were implanted with one PAMP-coated or PBS-coated monofilament. OD of each implant was quantified using fluorescence microscopy and imageJ software to measure the encapsulation response around the implants. Asterisks indicate $p < 0.05$ between coated implants ("PAMPlants" and control PBS-coated implants using Tukey HSD pairwise comparisons). All coated implants nearly doubled the immune response compared to the control. Sample sizes (*N*) indicate the number of bees singly implanted. *B13G* β-1,3-glucan (fungal PAMP); *LPS* lipopolysaccharide (Gram-negative bacteria PAMP); *PGN* peptidoglycan (Gram-positive bacteria PAMP)

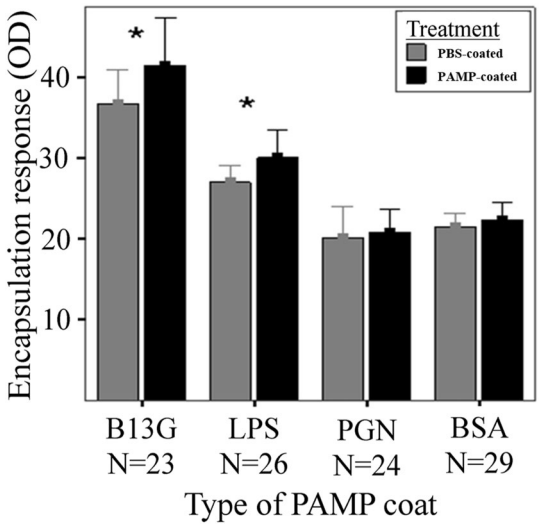


**Fig. 2** Mean encapsulation response (as measured via optical density, OD) of doubly implanted honey bees (*Apis mellifera*) ± 1 SE. Adult bees were doubly implanted with two nylon monofilaments. One monofilament was PBS-coated, while the other was coated in one of the four types of molecules: β-1,3-glucan (B13G), lipopolysaccharide (LPS), peptidoglycan (PGN), or bovine serum albumin (BSA). The optical density (OD) of implants was quantified using fluorescence microscopy and imageJ software to measure the encapsulation response around the implants. Asterisks indicate $p < 0.05$ between the PAMPlant and the internal control using paired $t$ tests. Sample sizes (*N*) indicate the number of bees doubly implanted

## Discussion

Using a simple technique, Metchnikoff discovered the process of ER in invertebrates resulting in a better understanding of cellular immunity and paving the way for modern advancements in assay methods (see Table 1). Building upon these approaches, we report results from a method for testing more specific immune function in a social insect. Our results suggest that the evolution of physiological immunity in insect societies is likely context dependent: both the relaxed selection hypothesis and the social group hypothesis could hold true, depending on the invader.

Our experimental honey bees exhibited a stronger ER when exposed to foreign bodies coated with molecules found on pathogens (i.e., PAMPs). As these PAMPs are conserved molecules found on and in the cell wall of bacteria and fungi (see Postel and Kemmerling (2009)), it is not surprising that hosts evolved to recognize PAMPs as

foreign cues, and induce an appropriate immune response to clear them. Uncoated implants are foreign and elicit an immune response (Table 1); however, our findings suggest that results gathered from uncoated or PBS-coated implants may not account for the specificity and/or degree of insect immune response.

When compared with control treatments, singly implanted individuals showed a greater immune response to all PAMPlants. The doubly implanted individuals exhibited a significantly stronger immune response to B13G and LPS when compared with internal controls. These data suggest that while Lopez-Uribe et al. (2016) found reduced ER to LPS in social insect lineages, physiological immunity is likely still important for these two classes of pathogens. B13G PAMPlants are a good candidate to build upon results found by Lopez-Uribe et al. (2016) as honey bees use both behavioral and physiological immunity to combat fungal invaders. Behavioral immunity is used to combat a common fungal pathogen, *Ascosphaera apis* (chalkbrood) (Starks et al. 2000), while physiological immunity is used to combat more virulent fungal diseases caused by *Aspergillus a*nd *Nosema* spp. (Glinski and Jaroz 2001; Li et al. 2017; Paxton et al. 2007).

There was no difference in ER for individuals doubly implanted with PGN and the PBS-coated implant. In accordance with our results, it has been shown that honey bees protect the colony from Gram-positive infections, such as *Paenibacillus larvae* (American foulbrood), via hygienic behavior (Spivak and Reuter 2001). Hence, in honey bees, as predicted by the social group hypothesis, social immunity may be more important than individual immunity when it comes to defending against Gram-positive pathogens. Alternatively, the lack of ER in response to PGN in doubly implanted individuals could stem from the evolutionary relationship between honey bees and their microbial symbionts. Worldwide, *Lactobacillus* and *Bifidobacterium* spp. are found in the gut microbiome of adult bees; both species are likely important for metabolism and immune function [Raymann and Moran (2018) and references therein].

Interestingly, the immune response to single implants was nearly double that of doubly implanted individuals. One possible explanation for this finding is that the immune response was divided between the two foreign bodies, albeit unevenly. Although these data were collected over two different years, we feel confident in this explanation as this uneven response was also observed by Allander and Schmid-Hempel (2000) in bumble bees. The bumble bee immune response to a primary immune challenge (either an implant or infection with *Crithida bombi*) was higher than the immune response to a second implant in the same individual. Our study is the first to show the possible division of the immune response in an individual with multiple, *simultaneous*, physical insults. Depending on the study system and the question being asked, this simultaneous internal control may be more ecologically relevant: honey bees can be plagued by multiple parasites and pathogens at the same time.

In contrast to the single implant trials, the data in our paired implant trials were not uniform. In the paired implant trials, honey bees showed a stronger response to fungi (B13G) over Gram-negative bacteria (LPS) and even more so over Gram-positive bacteria (PGN). We speculate that this makes sense both evolutionarily and ecologically; as mentioned earlier, colonies are commonly plagued by microsporidian *Apsergillus* and *Nosema* spp. (Glinski and Jaroz 2001; Li et al. 2017; Paxton et al. 2007).

Although our focus is on ER, the immune response is multi-faceted and insect societies have myriad weapons for thwarting disease. The ER mode of immunity is particularly important and is associated with physiological disease resistance to virally infected cells (Trudeau et al. 2001; Washburn et al. 1996), parasitoids (Carton and David 1983; Kraaijeveld et al. 2001), and parasites (Doums and Schmid-Hempel 2000). Indeed, the phenoloxidase (PO) cascade is similarly important, as it plays a complementary role in the ER process by contributing to resistance to bacteria (Ashida and Brey 1997; Pye 1974), fungi (Ochiai and Ashida 1988), viruses (Beck and Strand 2007; Wilson et al. 2001), parasites (Gorman et al. 1996; Leonard et al. 1985; Paskewitz and Riehle 1994), and parasitoids (Wilson et al. 2001). The single measure of immune function reported here (ER) has provided important information to researchers interested the evolutionary dynamics of host/pathogen relationships (Table 1).

Our PAMPlant technique enables researchers to explore the mechanism associated with defense against specific classes of microbes on host disease resistance. Data presented here confirm the specificity of the invertebrate immune system. Accordingly, understanding immune responses to historically common pathogens will be facilitated using biologically inspired techniques. Each study in Table 1 documents an opportunity to dig deeper and understand the degree to which immune function interacts with sex, behavior, dominance, mate choice, foraging ability, energetics, diet, habitat type, infection risk, co-infection, individual and colony condition, ontogeny, and invasion biology. Moreover, comparative studies using social species as a study system will help us better understand the evolution of disease resistance in insect societies, as well as the evolution of sociality.

**Acknowledgements** We are grateful for the helpful comments from Kelsey K. Graham, Stephanie Clarke, Rebecca Czaja. ET and LL were funded through NSF DBI 0649190 granted to PTS. We also thank two anonymous reviewers and the editor for their helpful comments and suggestions.

## References


Ahtiainen JJ, Alatalo RV, Kortet R, Rantala MJ (2004) Sexual advertisement and immune function in an arachnid species (Lycosidae). Behav Ecol 15:602–606. https://doi.org/10.1093/beheco/arh062

Allander K, Schmid-Hempel P (2000) Immune defence reaction in bumble-bee workers after a previous challenge and parasitic coinfection. Funct Ecol 14:711–717. https://doi.org/10.1046/j.1365-2435.2000.00476.x

Antunez K, Martin-Hernandez R, Prieto L, Meana A, Zunino P, Higes M (2009) Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). Envrion Microbiol 11:2284–2290

Appler RH, Frank SD, Tarpy DR (2015) Within-colony variation in the immunocompetency of managed and feral honey bees (*Apis mellifera* L.) in different urban landscapes. Insects 6:912–925. https://doi.org/10.3390/insects6040912

Ashida M, Brey P (1997) Recent advances in research on the insect phenoloxidase cascade. In: Brey P, Hultmark D (eds) Molecular mechanisms of immune responses in insects. Chapman and Hall, London, pp 135–172

Baer B, Schmid-Hempel P (2011) Unexpected consequences of polyandry for parasitism and fitness in the bumble bee, *Bombus terrestris*. Evolution 55:1639–1643

Beck G, Habicht GS (1996) Immunity and the invertebrates. Sci Am 275:60–66

Beck MH, Strand MR (2007) A novel polydnavirus protein inhibits the insect prophenoloxidase activation pathway. Proc Natl Acad Sci USA 104:19267–19272

Boete C, Paul REL, Koella JC (2002) Reduced efficacy of the immune melanization response in mosquitoes infected by malaria parasites. Parasitology 125:93–98. https://doi.org/10.1017/s0031182002001944

Brandt A, Gorenflo A, Siede R, Meixner M, Buchler R (2016) The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.). J Insect Physiol 86:40–47. https://doi.org/10.1016/j.jinsphys.2016.01.001

Calleri DV, Reid EM, Rosengaus RB, Vargo EL, Traniello JFA (2006) Inbreeding and disease resistance in a social insect: effects of heterozygosity on immnnocompetence in the termite *Zootermopsis angusticollis*. Proc R Soc B 273:2633–2640. https://doi.org/10.1098/rspb.2006.3622

Calleri DV, Rosengaus RB, Traniello JFA (2007) Immunity and reproduction during colony foundation in the dampwood termite *Zootermopsis angusticollis*. Physiol Entomol 32:136–142. https://doi.org/10.1111/j.1365-3032.2007.00559.x

Carton Y, David JR (1983) Reduction of fitness in *Drosophila* adults surviving parasitization by a cynipid wasp. Experientia 39:231–233

Chernyak L, Tauber AI (1988) The birth of immunology: Metchnikoff, the embryologist. Cell Immunol 117:218–233

Civantos E, Ahnesjo J, Forsman A (2005) Immune function, parasitization and extended phenotypes in colour polymorphic pygmy grasshoppers. Biol J Lin Soc 85:373–383. https://doi.org/10.1111/j.1095-8312.2005.00494.x

Contreras-Garduno J, Canales-Lazcano J, Cordoba-Aguilar A (2006) Wing pigmentation, immune ability, fat reserves and territorial status in males of the rubyspot damselfly, *Hetaerina americana*. J Ethol 24:165–173. https://doi.org/10.1007/s10164-005-0177-z

Daukste J, Kivleniece I, Krama T, Rantala MJ, Krams I (2012) Senescence in immune priming and attractiveness in a beetle. J Evolut Biol 25:1298–1304. https://doi.org/10.1111/j.1420-9101.2012.02516.x

de Souza DJ, van Vlaenderen J, Moret Y, Lenoir A (2008) Immune response affects ant trophallactic behaviour. J Insect Physiol 54:828–832. https://doi.org/10.1016/j.jinsphys.2008.03.001

Debecker S, Sommaruga R, Maes T, Stoks R (2015) Larval UV exposure impairs adult immune function through a trade-off with larval investment in cuticular melanin. Funct Ecol 29:1292–1299. https://doi.org/10.1111/1365-2435.12435

Di Lelio I et al (2014) Functional analysis of an immune gene of Spodoptera littoralis by RNAi. J Insect Physiol 64:90–97. https://doi.org/10.1016/j.jinsphys.2014.03.008

Doums C, Schmid-Hempel P (2000) Immunocompetence in workers of a social insect, *Bombus terrestris* L., in relation to foraging activity and parasitic infection. Can J Zool 78:1060–1066

Duong TM, McCauley SJ (2016) Predation risk increases immune response in a larval dragonfly (*Leucorrhinia intacta*). Ecology 97:1605–1610

Freitak D, Ots I, Vanatoa A, Horak P (2003) Immune response is energetically costly in white cabbage butterfly pupae. Proc R Soc B 270:S220–S222. https://doi.org/10.1098/rsbl.2003.0069

Gershman SN (2008) Sex-specific differences in immunological costs of multiple mating in *Gryllus vocalis* field crickets. Behav Ecol 19:810–815. https://doi.org/10.1093/beheco/arn040

Gherlenda AN, Haigh AM, Moore BD, Johnson SN, Riegler M (2016) Climate change, nutrition and immunity: effects of elevated $CO_2$ and temperature on the immune function of an insect herbivore. J Insect Physiol 85:57–64. https://doi.org/10.1016/j.jinsphys.2015.12.002

Gilbert R, Uetz GW (2016) Courtship and male ornaments as honest indicators of immune function. Anim Behav 117:97–103. https://doi.org/10.1016/j.anbehav.2016.04.013

Glinski Z, Jaroz J (2001) Infection and immunity in the honey bee, *Apis mellifera*. Apiacta 36:12–20

González M, Cost FG, Peretti AV (2015) Funnel-web construction and estimated immune costs in *Aglaoctenus lagotis* (Araneae: Lycosidae). J Arachnol 43:158–167

Gordon SM (2008) Elie Metchnikoff: father of natural immunity. J Eur Immunol 38:3257–3264

Gorman MJ, Cornel AJ, Collins FH, Paskewitz SM (1996) A shared genetic mechanism for melanotic encapsulation of CM-Sephadex beads and a malaria parasite, *Plasmodium cynomolgi* B, in the mosquito, *Anopheles gambiae*. Exp Parasitol 84:380–386

Haviola S, Kapari L, Ossipov V, Rantala MJ, Ruuhola T, Haukioja E (2007) Foliar phenolics are differently associated with *Epirrita autumnata* growth and immunocompetence. J Chem Ecol 33:1013–1023. https://doi.org/10.1007/s10886-007-9271-8

Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443:931–949

Hu QQ, Wei XH, Li YP, Wang JL, Liu XS (2017) Identification and characterization of a gene involved in the encapsulation response of *Helicoverpa armigera* haemocytes. Insect Mol Biol 26:752–762. https://doi.org/10.1111/imb.12336

Kang Y, Blanco K, Davis T, Wang Y, DeGrandi-Hoffman G (2016) Disease dynamics of honeybees with *Varroa destructor* as parasite and virus vector. Math Biosci 275:71–92

Kangassalo K, Kosonen K, Pölkki M, Sorvari J, Krams I, Rantala MJ (2016) Immune challenge has a negative effect on cuticular darkness in the mealworm beetle, *Tenebrio molitor*. Annales Zoologici Fennici 53:255–262

Kapari L, Haukioja E, Rantala MJ, Ruuhola T (2006) Defoliating insect immune defense interacts with induced plant defense during a population outbreak. Ecology 87:291–296. https://doi.org/10.1890/05-0362

Kelly CD (2016) Effect of nutritional stress and sex on melanotic encapsulation rate in the sexually size dimorphic Cook Strait

giant weta (*Deinacrida rugosa*). Can J Zool 94:787–792. https://doi.org/10.1139/cjz-2016-0108

Kelly CD (2017) Sexually dimorphic effect of mating on the melanotic encapsulation response in the harem-defending Wellington tree weta, *Hemideina crassidens* (Orthoptera: Tettigonioidea: Anostostomatidae). Ethology 123:785–792. https://doi.org/10.1111/eth.12650

Kelly CD, Jennions MD (2009) Sexually dimorphic immune response in the harem polygynous Wellington tree weta *Hemideina crassidens*. Physiol Entomol 34:177–179

Kirschman LJ, Quade AH, Zera AJ, Warne RW (2017) Immune function trade-offs in response to parasite threats. J Insect Physiol 98:199–204. https://doi.org/10.1016/j.jinsphys.2017.01.009

Kivleniece I, Krams I, Daukste J, Krama T, Rantala MJ (2010) Sexual attractiveness of immune-challenged male mealworm beetles suggests terminal investment in reproduction. Anim Behav 80:1015–1021. https://doi.org/10.1016/j.anbehav.2010.09.004

Klemola N, Klemola T, Rantala MJ, Ruuhola T (2007) Natural host-plant quality affects immune defence of an insect herbivore. Entomologia Experimentalis Et Applicata 123:167–176. https://doi.org/10.1111/j.1570-7458.2007.00533.x

Konig C, Schmid-Hempel P (1995) Foraging activity and immunocompetence in workers of the bumble bee, *Bombus terrestris* L. Proc R Soc B 260:225–227. https://doi.org/10.1098/rspb.1995.0084

Koskimaki J, Rantala MJ, Taskinen J, Tynkkynen K, Suhonen J (2004) Immunocompetence and resource holding potential in the damselfly, *Calopteryx virgo* L. Behav Ecol 15:169–173. https://doi.org/10.1093/beheco/arg088

Kimbrell DA, Beutler B (2001) The evolution and genetics of innate immunity. Nat Rev Genet 2:256–267

Kraaijeveld AR, Limentani EC, Godfray HCJ (2001) Basis of the trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. Proc R Soc B Biol Sci 268:259–261

Krams I, Daukste J, Kivleniece I, Krama T, Rantala MJ (2013) Previous encapsulation response enhances within individual protection against fungal parasite in the mealworm beetle *Tenebrio molitor*. Insect Sci 20:771–777. https://doi.org/10.1111/j.1744-7917.2012.01574.x

Krams I et al (2015a) Effects of food quality on trade-offs among growth, immunity and survival in the greater wax moth *Galleria mellonella*. Insect Sci 22:431–439. https://doi.org/10.1111/1744-7917.12132

Krams IA, Krama T, Trakimas G, Kaasik A, Rantala MJ, Škute A (2015b) Reproduction is costly in an infected aquatic insect. Ethol Ecol Evolut 29:74–84. https://doi.org/10.1080/03949370.2015.1089943

Krams I et al (2016) A dark cuticle allows higher investment in immunity, longevity and fecundity in a beetle upon a simulated parasite attack. Oecologia 182:99–109. https://doi.org/10.1007/s00442-016-3654-x

Krams IA et al (2017) Microbiome symbionts and diet diversity incur costs on the immune system of insect larvae. J Exp Biol 220:4204–4212. https://doi.org/10.1242/jeb.169227

Lee KP, Cory JS, Wilson K, Raubenheimer D, Simpson SJ (2006) Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. Proc R Soc B 273:823–829. https://doi.org/10.1098/rspb.2005.3385

Leonard C, Ratcliffe NA, Rowley AF (1985) The role of prophenoloxidase activation in non-self recognition and phagocytosis by insect blood cells. J Insect Physiol 31:789–799

Li W, Evans JD, Li J, Su S, Hamilton M, Chen Y (2017) Spore load and immune response of honey bees naturally infected by *Nosema ceranae*. Parasitol Res 116:3265–3274

Li LF, Xu ZW, Liu NY, Wu GX, Ren XM, Zhu JY (2018) Parasitism and venom of ectoparasitoid *Scleroderma guani* impairs host cellular immunity. Arch Insect Biochem Physiol 98 https://doi.org/10.1002/arch.21451

Lopez-Uribe MM, Sconiers WB, Frank SD, Dunn RR, Tarpy DR (2016) Reduced cellular immune response in social insect lineages. Biol Lett 12:20150984 https://doi.org/10.1098/rsbl.2015.0984

Mariani F et al (2012) Parasitic interactions between *Nosema* spp. and *Varroa destructor* in *Apis mellifera* colonies. Zoo Trop 30:81–90

Martin SJ (2001) The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. J Appl Ecol 38:1082–1093

Moreno-Garcia M, Cordoba-Aguilar A, Conde R, Lanz-Mendoza H (2013) Current immunity markers in insect ecological immunology: assumed trade-offs and methodological issues. B Entomol Res 103:127–139

Negri P, Quintana S, Maggi M, Szawarski N, Lamattina L, Eguaras M (2014) Apis mellifera hemocytes generate increased amounts of nitric oxide in response to wounding/encapsulation. Apidologie 45:610–617. https://doi.org/10.1007/s13592-014-0279-0

Nurnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev 198:249–266

Ochiai M, Ashida M (1988) Purification of a B1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of the silkworm, *Bombyx mori*. J Biol Chem 263:12056–12062

Paskewitz S, Riehle MA (1994) Response of plasmodium refractory and susceptible strains of *Anopheles gambiae* to inoculated sephadex beads. Dev Comp Immunol 18:369–375

Paxton RJ, Klee J, Korpela S, Fries I (2007) *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie 38:558–565

Pereira KD, Guedes NMP, Serrao JE, Zanuncio JC, Guedes RNC (2017) Superparasitism, immune response and optimum progeny yield in the gregarious parasitoid *Palmistichus elaeisis*. Pest Manag Sci 73:1101–1109. https://doi.org/10.1002/ps.4534

Polkki M, Kangassalo K, Rantala MJ (2014) Effects of interaction between temperature conditions and copper exposure on immune defense and other life-history traits of the blow fly *Protophormia terraenovae*. Environ Sci Technol 48:8793–8799. https://doi.org/10.1021/es501880b

Pomfret JC, Knell RJ (2006) Immunity and the expression of a secondary sexual trait in a horned beetle. Behav Ecol 17:466–472. https://doi.org/10.1093/beheco/arj050

Postel S, Kemmerling B (2009) Plant systems for recognition of pathogen-associated molecular patterns. Semin Cell Dev Biol 20:1025–1031

Prokkola J, Roff D, Karkkainen T, Krams I, Rantala MJ (2013) Genetic and phenotypic relationships between immune defense, melanism and life-history traits at different temperatures and sexes in *Tenebrio molitor*. Heredity 111:89–96. https://doi.org/10.1038/hdy.2013.20

Pye AE (1974) Microbial activation of prophenoloxidase from immune insect larvae. Nature 251:610–613

Rantala MJ, Kortet R (2003) Courtship song and immune function in the field cricket *Gryllus bimaculatus*. Biol J Linn Soc 79:503–510. https://doi.org/10.1046/j.1095-8312.2003.00202.x

Rantala MJ, Kortet R (2004) Male dominance and immunocompetence in a field cricket. Behav Ecol 15:187–191. https://doi.org/10.1093/beheco/arg103

Rantala MJ, Roff DA (2005) An analysis of trade-offs in immune function, body size and development time in the Mediterranean field cricket *Gryllus bimaculatus*. Funct Ecol 19:323–330. https://doi.org/10.1111/j.1365-2435.2005.00979.x

Rantala MJ, Jokinen I, Kortet R, Vainikka A, Suhonen J (2002) Do pheromones reveal male immunocompetence? Proc R Soc B 269:1681–1685. https://doi.org/10.1098/rspb.2002.2056

Rantala MJ, Vainikka A, Kortet R (2003) The role of juvenile hormone in immune function and pheromone production trade-offs: a test of the immunocompetence handicap principle. Proc R Soc B 270:2257–2261. https://doi.org/10.1098/rspb.2003.2472

Rantala MJ, Viitaniemi H, Roff DA (2011) Effects of inbreeding on potential and realized immune responses in *Tenebrio molitor*. Parasitology 138:906–912. https://doi.org/10.1017/S0031182011000473

Raymann K, Moran NA (2018) The role of the gut microbiome in health and disease of adult honey bee workers. Curr Opin Insect Sci 26:97–104

Robb T, Jamieson IG, Forbes MR (2003) Morphs and immunity of the alpine weta, *Hemideina maora*: influence of temperature, selection, and associations with density and cuticular melanism. Ecol Entomol 28:738–746

Ryder JJ, Siva-Jothy MT (2000) Male calling song provides a reliable signal of immune function in a cricket. Proc R Soc B 267:1171–1175. https://doi.org/10.1098/rspb.2000.1125

Sadd BM, Siva-Jothy MT (2006) Self-harm caused by an insect's innate immunity. Proc R Soc B 273:2571–2574. https://doi.org/10.1098/rspb.2006.3574

Sammataro D, Gerson U, Needham G (2000) Parasitic mites of honey bees: life history, implications, and impact. Annu Rev Entomol 45:519–548

Schmid-Hempel R, Schmid-Hempel P (1998) Colony performance and immunocompetence of a social insect, *Bombus terrestris* in poor and variable environments. Funct Ecol 12:22–30

Silva FWS, Elliot SL (2016) Temperature and population density: interactional effects of environmental factors on phenotypic plasticity, immune defenses, and disease resistance in an insect pest. Ecol Evolut 6:3672–3683. https://doi.org/10.1002/ece3.2158

Siva-Jothy MT (2000) A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. Proc Roy Soc B Biol Sci 267:2523–2527. https://doi.org/10.1098/rspb.2000.1315

Smilanich AM, Dyer LA, Gentry GL (2009) The insect immune response and other putative defenses as effective predictors of parasitism. Ecology 90:1434–1440. https://doi.org/10.1890/08-1906.1

Smith VJ (2016) Immunology of invertebrates: cellular. In: Delves P (ed) Encyclopedia of life science. Wiley, New York

Sorvari J, Hakkarainen H, Rantala MJ (2008) Immune defense of ants is associated with changes in habitat characteristics. Environ Entomol 37:51–56. https://doi.org/10.1603/0046-225x(2008)37%5B51:idoaia%5D2.0.co;2

Sorvari J, Rantala LM, Rantala MJ, Hakkarainen H, Eeva T (2007) Heavy metal pollution disturbs immune response in wild ant populations. Environ Pollut 145:324–328. https://doi.org/10.1016/j.envpol.2006.03.004

Spivak M (1996) Honey bee hygienic behavior and defense against *Varroa jacobsoni*. Apidologie 27:245–260

Spivak M, Reuter GS (2001) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. Apidologie 32:555–565

Srygley RB, Jaronski ST (2018) Protein deficiency lowers resistance of Mormon crickets to the pathogenic fungus *Beauveria bassiana*. J Insect Physiol 105:40–45. https://doi.org/10.1016/j.jinsphys.2018.01.005

Srygley RB, Lorch PD (2011) Weakness in the band: nutrient-mediated trade-offs between migration and immunity of Mormon crickets *Anabrus simplex*. Anim Behav 81:395–400. https://doi.org/10.1016/j.anbehav.2010.11.006

Srygley RB, Lorch PD, Simpson SJ, Sword GA (2009) Immediate protein dietary effects on movement and the generalised immunocompetence of migrating Mormon crickets Anabrus simplex (Orthoptera: Tettigoniidae). Ecol Entomol 34:663–668. https://doi.org/10.1111/j.1365-2311.2009.01117.x

Starks PT, Blackie CA, Seeley TD (2000) Fever in honey bee colonies. Naturwissenschaften 87:229–231

Tauber AI (2003) Metchnikoff and the phagocytosis theory. Nat Rev Mol Cell Biol 4:897–901

Teng ZW, Xu G, Gan SY, Chen X, Fang Q, Ye GY (2016) Effects of the endoparasitoid *Cotesia chilonis* (Hymenoptera: Braconidae) parasitism, venom, and calyx fluid on cellular and humoral immunity of its host *Chilo suppressalis* (Lepidoptera: Crambidae) larvae. J Insect Physiol 85:46–56. https://doi.org/10.1016/j.jinsphys.2015.11.014

Trudeau D, Washburn JO, Volkman LE (2001) Central role of hemocytes in *Autographa californica M* nucleopolyhedrovirus pathogenesis in *Heliothis virescens* and *Helicoverpa zea*. J Virol 75:996–1003

Vainikka A, Rantala MJ, Seppala O, Suhonen J (2007) Do male mealworm beetles, *Tenebrio molitor*, sustain the honesty of pheromone signals under immune challenge? Acta Ethol 10:63–72. https://doi.org/10.1007/s10211-007-0031-0

Vainio L, Hakkarainen H, Rantala MJ, Sorvari J (2004) Individual variation in immune function in the ant *Formica exsecta*; effects of the nest body size sex. Evolut Ecol 18:75–84. https://doi.org/10.1023/B:EVEC.0000017726.73906.b2

van Engelsdorp D, Meixner MD (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J Invertebr Pathol 103:S80–S95

Vanikova S, Noskova A, Pristas P, Judova J, Javorsky P (2015) Heterotrophic bacteria associated with *Varroa destructor* mite. Apidologie 46:369–379

Wang P et al (2017) C-type lectin interacting with beta-integrin enhances hemocytic encapsulation in the cotton bollworm, *Helicoverpa armigera*. Insect Biochemistry Molecular Biology 86:29–40. https://doi.org/10.1016/j.ibmb.2017.05.005

Washburn JO, Kirkpatrick BA, Volkman LE (1996) Insect protection against viruses. Nature 383:767–767

Wilson EO (1971) The insect societies. Harvard University Press, Cambridge

Wilson EO (1975) Sociobiology: the new synthesis. Harvard University Press, Cambridge

Wilson K, Cotter SC, Reeson AF, Pell JK (2001) Melanism and disease resistance in insects. Ecol Lett 4:637–649

Wilson K, Thomas MB, Blanford S, Doggett M, Simpson SJ, Moore SL (2002) Coping with crowds: density-dependent disease resistance in desert locusts. Proc Natl Acad Sci 99:5471–5475. https://doi.org/10.1073/pnas082461999

Wilson-Rich N, Starks PT (2010) The *Polistes* war: weak immune function in the invasive *Polistes dominulus* relative to the natie *P. fuscatus*. Insectes Soc 57:47–52

Wootton EC, Dyrynda EA, Ratcliffe NA (2006) Interaction between non-specific electrostatic forces and humoral factors in haemocyte attachment and encapsulation in the edible cockle *Cerastoderma edule*. J Exp Biol 209:1326–1335. https://doi.org/10.1242/jeb.02118

Wilson-Rich N, Dres ST, Starks PT (2008) The ontogeny of immunity: development of innate immune strength in the honey bee (*Apis mellifera*). J Insect Physiol 54:1392–1399. https://doi.org/10.1016/j.jinsphys.2008.07.016

Wilson-Rich N, Spivak M, Fefferman NH, Starks PT (2009) Genetic, individual, and group facilitation of disease resistance in insect societies. Annu Rev Entomol 54:405–423

Wilson-Rich N, Hester F, Pilowsky JA, Foo B, Tien T, Starks PT (2014) A test of the haploid susceptibility hypothesis using a

species with naturally occurring variation in ploidy. Insectes Soc 61:163–169

Winston ML (1987) The biology of the honey bee. Harvard University Press, Cambridge

Wu M et al (2014) Inhibitory effect of gut bacteria from the Japanese honey bee, *Apis cerana japonica*, against *Melissococcus plutonius*, the causal agent of European foulbrood disease. J Insect Sci 14:1–13

Yaroslavtseva ON, Dubovskiy IM, Khodyrev VP, Duisembekov BA, Kryukov VY, Glupov VV (2017) Immunological mechanisms of synergy between fungus *Metarhizium robertsii* and bacteria *Bacillus thuringiensis* ssp. *morrisoni* on Colorado potato beetle larvae. J Insect Physiol 96:14–20. https://doi.org/10.1016/j.jinsphys.2016.10.004

Zhong K, Liu ZC, Wang JL, Liu XS (2017) The entomopathogenic fungus *Nomuraea rileyi* impairs cellular immunity of its host *Helicoverpa armigera*. Arch Insect Biochem Physiol. https://doi.org/10.1002/arch.21402

Zhuo XR et al (2018) 20-Hydroxyecdysone promotes release of GBP-binding protein from oenocytoids to suppress hemocytic encapsulation. Insect Biochem Mol Biol 92:53–64. https://doi.org/10.1016/j.ibmb.2017.11.006

Zipfel C, Felix G (2005) Plants and animals: a different taste for microbes? Curr Opin Plant Biol 8:353–360