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Immunosenescence and resistance to parasite infection in the honey bee, *Apis mellifera*

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ABSTRACT

Immunosenescence, the systemic reduction of immune efficiency with age, is increasingly recognised as having important implications for host-parasite dynamics. Changes in the immune response can impact on the ability of an individual to resist or moderate parasite infection, depending on how and when it encounters a parasite challenge. Using the European honey bee *Apis mellifera* and its microsporidian parasite *Nosema ceranae*, we investigated the effects of host age on the ability to resist parasite infection and on baseline immunocompetence, assessed by quantifying constitutive (PO) and potential levels (PPO) of the phenoloxidase immune enzyme as general measures of immune function. There was a significant correlation between the level of general immune function and infection intensity, but not with survival, and changes in immune function with age correlated with the ability of individuals to resist parasite infection. Older individuals had better survival when challenged with a parasite than younger individuals, however they also had more intense infections and lower baseline immunocomptence. The ability of older individuals to have high infection intensities yet live longer, has potential consequences for parasite transmission. The results highlight the need to consider age in host-parasite studies and show the importance of choosing the correct measure when assaying invertebrate immunity.

1. Introduction

Why organisms grow old and die is a fundamental question in biology (Kirkwood and Austad, 2000). In an experimental context, aging of individuals is often viewed as an investigational nuisance, which is either excluded or overlooked. However, when investigated explicitly, aging has proved to not just be a random decline in physiological functioning, but a progressive, pre-programmed decline in homeostatic systems and fitness (Münch et al., 2008). This senescence of fitness components with increasing age occurs in many organisms, affecting various life-history traits, such as reproductive success, learning and foraging (Partridge, 1987; Gaillard et al., 2000; Tofilski, 2000; Catry et al., 2006).

Parasites are a universal threat to organisms and it has been suggested that parasite loads may increase with the age of the host, either due to an accumulation of parasites over time or a reduction in immunocompetence with age (Wilson et al., 2001). This is potentially significant for host-parasite population dynamics as it could create an aggregation of parasites in the older subset of the host population. Understanding what dynamics drive any accumulation of parasites with host age needs an understanding of the interaction between the parasite and the ability of hosts to resist infection. There is, however, conflicting empirical evidence for whether immune function continuously declines with age. Declines in immunocompetence as a function of age have been reported in both vertebrates and invertebrates (Adamo et al., 2001; Cichoń et al., 2003; Torroba and Zapata, 2003; Amdam et al., 2005; Müller et al., 2013). Other studies, though, have found no difference or even an increase in immune function with age in both vertebrates and invertebrates (Rolff, 2001; Pletcher et al., 2002; Madsen and Ujvari, 2006; Wilson-Rich et al., 2008; Armitage and Boomsma, 2010). The impact of age on immunocompetence is therefore still not fully understood and probably depends on both the time after challenge and the immune variable measured.

Immune defences are costly which can lead to trade-offs and therefore temporal variations in responses (Sheldon and Verhulst, 1996). Responses can also vary depending upon the cause and strength of immune system activation (Korner and Schmid-Hempel, 2004; Haine et al., 2008). This creates complicated dynamics between changes in immune system with host age, and also with time and duration of activation, which are often confounded and overlooked in experimental design. The

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relationships between a measure of immune function and resistance to parasites are often not straightforward (Adamo, 2004b,a), and the age-immunity-resistance relationship may be particularly complex (Bull et al., 2012), but empirical studies of this are lacking.

Here we examine the effect of age on immunocompetence and susceptibility to infection, using the European honey bee, *Apis mellifera*, and its microsporidian parasite *Nosema ceranae*. Honey bees are an interesting system to study the interacting effects on immunocompetence because their immune system differs with age, social context, caste and task (Amdam et al., 2005; Münch et al., 2008; Wilson-Rich et al., 2008). In this experiment, we exposed laboratory-reared worker bees at fixed social densities to a controlled dose of parasite, to control for social context, caste and task, as well as parasite exposure, enabling any effect of age to be distinguished from the other social effects that typify the colony environment and which would otherwise confound or add noise to the results. We use *N. ceranae* as the experimental parasite. This is an obligate, intracellular, microsporidian parasite that infects adult honey bees and bumblebees through ingestion of mature spores that germinate and infect through the gut epithelium, and which has recently been associated with significant colony losses in some, but not all, regions (Higes et al., 2006; Klee et al., 2007; Higes et al., 2008; Paxton et al., 2008; Fries, 2010; Graystock et al., 2013a,b; Furst et al., 2014). The mechanisms of resistance to the *Nosema* species are unknown, and our aim was to determine the effects of age and *N. ceranae* infection on general immunocompetence, rather than to directly study immunity to *N. ceranae*. As our measure of general host immunocompetence we quantified levels of the immune enzyme phenoloxidase (PO), a constitutive response, and its precursor prophenoloxidase (PPO) as a measure of a potential immune response for each individual. The phenoloxidase cascade is an important, general immune response to a wide variety of parasites and thus provides an indication of the general immune status of an insect (Cotter and Wilson, 2002; Adamo, 2004a; Bocher et al., 2007; Haine et al., 2008).

2. Materials and methods

We investigated the immunocompetence and resistance to the *N. ceranae* parasite of bees that were 1, 2, 3, 8, 13 or 14 days old when exposed. Both bees and the *N. ceranae* parasite were obtained in June/July from a single apiary in West Yorkshire, UK, that contained 50 colonies of *A. mellifera carnica* honey bees. The infection status of colonies with *N. ceranae* was determined by screening individually the ventriculi of 60 adult bees from each colony, collected from the hive entrance, using conventional PCR (see below). Our previous work indicated that this sample size, in combination with the sensitive detection method of PCR, was sufficient to reliably determine the general infection status of a colony, and the effectiveness of this protocol was supported by the fact that none of the control bees in the experiment developed *N. ceranae* infections (see Results). To provide bees for the experiment, we selected six colonies (Am1128, Am1113, Am1123, Am1139, Am1138, and Am1111) in which we did not detect either *Nosema* species. We obtained a suspension of *N. ceranae* spores by homogenising the ventriculi of adult bees from an infected colony. Ventriculi were homogenised individually in 500 μl of molecular grade water, *Nosema* infection confirmed by microscopy, and the identity of the parasite (*N. ceranae* or *N. apis*) identified by PCR (see below). Spore suspensions that were positive for *N. ceranae* from 20 workers from each of 5 colonies were pooled and purified by Percoll centrifugation. Spore viability was checked using 10% Trypan blue dye staining (Antúnez et al., 2009), and a final *N. ceranae* suspension produced with 2500 viable spores per μl in a 50% sterile sucrose solution.

2.1. Molecular methods

To confirm the infection status of bees prior to the experiment, DNA was extracted from homogenised ventriculi by incubating with Proteinase K (Promega) (5 μl/ml) at 56 °C for 12 h before being boiled with 75 μl of 5% Chelex 100 (Biorad) suspended in 10 μM Tris Buffer for 15 min. DNA was amplified using primers specific for *N. ceranae* and *N. apis* (Chen et al., 2008), with reactions containing 1 μl DNA, 0.2 ml of each forward and reverse primer, 2 μl PCR buffer, 0.05 μl of 5 U/ml Taq and primer specific quantities of 25 mM $MgCl_2$ and 10 mM dNTPs, made up to 10 μl with ddH_20. Amplifications were performed in ABI3700 thermal cyclers with an initial denaturation of 1 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C, and a final extension step of 72 °C for 7 min. The mitochondrial *CO1* host control gene was amplified to confirm DNA quality using the *LCO-Hym*/*HCOout* primers, with 1.5 ml $MgCl_2$ and 1 μl dNTPs, with an initial denaturation of 2 min at 94 °C followed by 35 cycles of 30 seconds at 94 °C, 45 seconds at 50 °C and 2 min at 72 °C, and a final extension step of 72 °C for 7 min (Evison et al., 2012). We then used Taqman real-time PCR to further confirm the infection status of cases which appeared to be single infections of *N. ceranae*. Reactions were carried out in an ABI StepOne Plus thermal cycler using Taqman® Universal Master Mix with UGA (ABI). Primers, probe and reaction conditions for both *N. ceranae* and *N. apis* were as described previously (Bourgeois et al., 2010). The *A. mellifera β-actin* gene was used as an internal host control using published primers (Lourenço et al., 2008), that were modified for use with Taqman® by the design of an Molecular-Groove Binding Non-fluorescence Quencher (MGBNFQ) probe (NED-MGBNFQ- AAT TAA GAT CAT CGC GCC AC) using the Primer 3 program.

2.2. Experimental procedure

To obtain bees for the experiments, frames of brood were removed from the *Nosema*-free colonies and allowed to eclose in an incubator at 34 °C, 60% relative humidity (RH), with eclosing workers collected from the comb daily and kept in same-age, nestmate cohorts. These were organised such that for each of the six colonies there were four groups of 25 bees that were 1, 2, 3, 8, 13 or 14 days old on the day of experimental treatment. Two groups from each colony (two pots, each of 25 individuals) were randomly assigned to the treatment and the other two groups from each colony to the control. Bees were starved for one hour, then placed individually in harnesses made from 0.5 ml Eppendorf tubes and hand fed with 4 μl of either a 50% sucrose solution containing 10,000 viable *N. ceranae* spores or sterile sucrose solution control. Any bees that did not fully ingest the dose were discarded and replaced. Bees were then held individually for 1 h before being replaced in their groups to reduce any trophallactic sharing of the ingested dose. The groups of bees were kept in clean, plastic pots (11 cm diameter, Ambican 16 oz Fabrikal Delipot) at 34 °C and 60% RH, with an *ad libitum* supply of sterile 50% sucrose solution for the 12 day duration of the experiment. Survival was monitored daily, with dead bees stored in 90% ethanol at −20 °C. Two random individuals from each group were collected at 1, 2, 5, 8, 10 and 12 days after treatment for quantification of immunocompetence (see below). The intensity of *N. ceranae* infections in all bees was then determined by counting of the number of spores present in the hindgut using FastRead disposable haemocytometers (Immune Systems).

2.3. Immunocompetence assay

The bees collected for quantification of immunocompetence were anaesthetised on ice and haemolymph extracted by perfusion bleeding using 0.5 ml of ice-cold sodium cacodylate buffer (0.01 M

sodium cacodylate, 0.005 M $CaCl_2$, pH 6.5), with care taken to prevent rupture of the gut contents (Laughton et al., 2011). Samples were immediately snap frozen in liquid-nitrogen (−90 °C) to disrupt haemocytes and then stored at −20 °C. Prior to processing, samples were defrosted on ice, vortexed, and centrifuged at 4 °C for 20 min at 80,000 g to remove cell debris. The immune assays were carried out in microtitre plates prepared on ice. Haemolymph samples of 20 μl had 5 μl of PBS or 5 μl α-chymotrypsin (5 mg/ml^{-1} in ice cold ddH_20) added for PO or PPO analyses respectively, together with 180 μl ice cold assay master mix, made up of 135 μl of ddH_2O, 20 μl of PBS and 20 μl of saturated L-dopa (ice cold ddH_20, ~11 mg/ml^{-1}).

Activity of PPO and PO was performed for each individual bee sample simultaneously, with treatments split across plates. Four technical replicates were carried out for each haemolymph sample and each plate contained both blank samples, made up to the correct volume with 20 μl PBS, and four PO and PPO technical replicates of a positive control reference sample (made from the pooled haemolymph of 30 bees from each of four colonies) to control for variation across microtitre plates. Prepared plates were immediately placed in a pre-heated VersaMax microplate reader (Molecular Devices) at 28 °C, and reactions were allowed to proceed for one hour, with readings taken at 492 nm every 15 s. Mean levels of PO and PPO in each sample were calculated using Softmax Pro v5.0.1, with the reference samples used to correct for variation between plates. Technical replicates with $R^2 < 0.9$ in the linear section of the absorbance curve were removed; there were still at least two technical replicates for all samples in the final dataset from which a mean could be calculated.

2.4. Statistical analysis

All analyses were carried out in R 2.14.2 (R Development Core Team, 2009). The effects of treatment, age and their interaction on the survival of bees were determined using the *survreg* function of the *survival* package, with the *frailty* function used to fit colony as a random term in the survival models (Therneau, 2008). The hazard ratios of exposure to *N. ceranae* were extracted from survival models by comparison of the *N. ceranae* exposed bees to the control bees, for each age cohort. We analysed PO and PPO levels separately for control and treated bees, using linear mixed effects models with the *lmer* function of the *lme4* package (Bates and Maechler, 2010), with age and days after parasite exposure as factors, colony fitted as a random term, and days post-exposure included as a covariate (because bees were sampled for PO/PPO analysis across a time series following exposure). Spore counts from treated bees (no control bees had spores) were also analysed with linear mixed effects models, with age as a factor and colony fitted as a random term. All models were checked for normality of errors, and homogeneity of variance, through inspections of plots of residuals against fitted values and quantile–quantile plots. Models with the fixed effects were compared to the null models containing only the random terms in order to assess the validity of the mixed effects analyses, and therefore also generate significance; rejecting models which included fixed effects that did not differ significantly from the null model. Correlations between infection intensity (spore count) and immune function (PO and PPO) were investigated using Spearmans rank correlations.

3. Results

3.1. Survival

There was a significant interaction between the effects of treatment and age on the survival of bees ($\chi^2_7 = 74.2$, $P < 0.001$). Treated bees in general died at a faster rate than control bees, and the effect of parasite exposure on survival was greater for bees that were 1–3 days of age on exposure than those which were 8–14 days of age (Fig. 1a, Fig. S1). Younger bees generally survived better than older bees, and this effect of age was significant for both control and parasite-exposed bees when they were analysed separately ($\chi^2_9 = 133.6$, $P < 0.001$, and $\chi^2_9 = 58.6$, $P < 0.001$, respectively).

3.2. Immunocompetence

Levels of both PPO and, particularly, PO were highly variable, and the effects on them of treatment, age of bees or time post-exposure were complex (Fig. 2 and Table S1). There were significant interactions between the effects of treatment and age of bees, and treatment and time post-exposure on levels of PPO ($\chi^2_{14,11} = 23.2$, $P < 0.001$, and $\chi^2_{14,11} = 57.5$, $P < 0.001$, respectively). The level of PPO was significantly lower in bees exposed to *N. ceranae* than in control bees, even only 1 day after exposure ($\chi^2_{4,1} = 17.5$, $P < 0.001$, Fig. 2f–j). In *N. ceranae*-exposed bees, PPO levels were affected significantly by the age of bees ($\chi^2_{5,\ 8} = 21.8$, $P < 0.001$), appearing to be generally higher in younger bees (Fig. 2f–j), whereas age of bees had less effect on PPO levels in control bees, although levels were still consistently lowest in the oldest

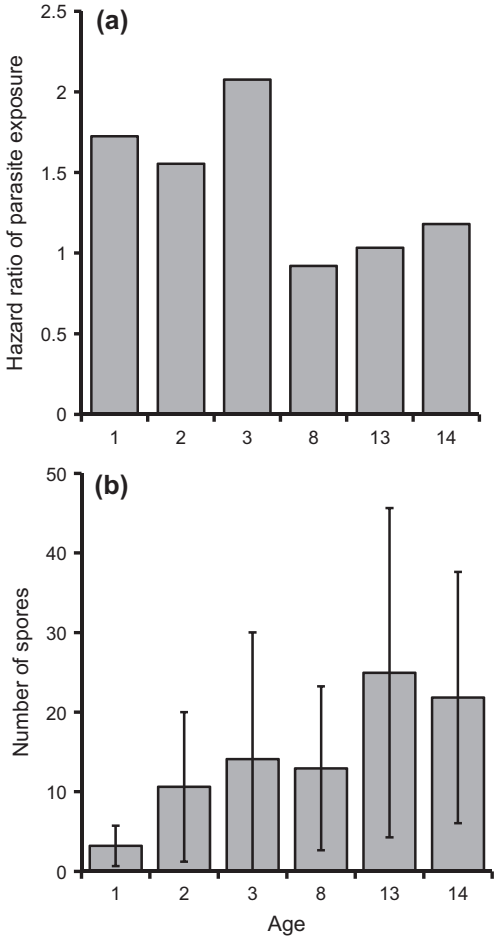

Fig. 1. (a) Hazard ratio of *Apis mellifera* exposure to the *N. ceranae* parasite relative to the control treatment, for that age and colony cohort, for honey bee workers that were exposed to the parasite at different ages (days) ($n = 150$ per age). (b) Mean (±s.e.) number of spores ($\times 10^6$) of the *N. ceranae* parasite in the ventriculi of parasite exposed individuals ($n = 150$ per age). The hazard of exposure was calculated from survival over the 12 day period following exposure and parasite spores were counted at the end of this 12 day period (for full survival curves, see Supplementary information Fig S1).

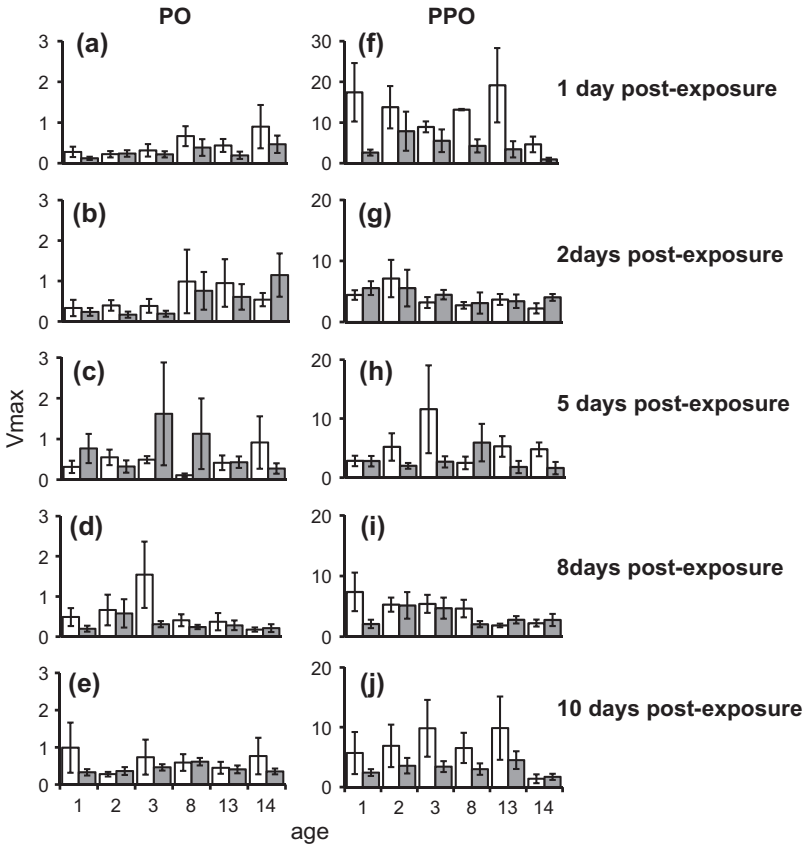

Fig. 2. Mean ± s.e. Enzyme activity, the V_{max}, is the change in absorbance over time during the linear phase of the enzymatic reaction for phenoloxidase (PO; a–e) and prophenoloxidase (proPO; f–j) immune enzymes in honey bees of different ages (days) that were either exposed to the *N. ceranae* parasite (grey columns) or control solution (white columns), either one (a and f), two (b and g), five (c and h), eight (d and i) or ten (e and j) ($n = 12$ per age) days after treatment.

bees ($\chi^2_{5,8} = 6.01$, $P = 0.304$). PPO levels in *N. ceranae*-exposed bees were relatively similar across different times after exposure, whereas PPO levels in control bees were generally higher 1 day after exposure than later. PO levels were much lower and more variable still than PPO levels (Fig. 2a–e). PO levels were affected significantly by the interaction between treatment and time post-exposure ($\chi^2_{11,14} = 21.8$, $P = 0.02$), with levels being higher in *N. ceranae*-exposed than control bees 5 days post-exposure, but generally similar or lower on other days post-exposure (Fig. 2a–e). PO levels were not affected significantly by the age of bees ($\chi^2_{5,\ 8} = 5.18$, $P = 0.39$).

3.3. Parasite infection intensity

None of the control bees from any colony or cohort were found to be infected by *N. ceranae*. The number of *N. ceranae* spores present in the ventriculi of treated bees was significantly affected by age ($\chi^2_{1,6} = 17.01$, $P < 0.001$), with bees that were older when exposed tending to have more spores in their hindguts at the end of the experiment than bees that were younger when exposed (Fig. 1b). For example, individuals that were 13 or 14 days old when exposed had over 2×10^6 spores in their ventriculi on average at the end of the experiment, whereas bees that were only one day old when exposed had approximately ten times fewer spores, with 0.2×10^6 spores on average at the end of the experiment.

3.4. Parasite load and immune measure correlations

There appeared to be slightly positive relationships between the survival of bees and their immune enzyme levels, but these relationships were not significant (PO: $\rho = 0.165$, $N = 35$, $P = 0.362$, Fig. 3b; PPO: $\rho = 0.0182$, $N = 35$, $P = 0.318$, Fig. 3a). There was, however, a significant negative relationship between the number of *N. ceranae* spores in bees and their levels of PPO ($\rho = -0.482$, $N = 35$, $P = 0.005$, Fig. 3c), and a similar, but non-significant, trend for the relationship between *N. ceranae* spores and PO levels ($\rho = -0.031$, $N = 35$, $P = 0.862$, Fig. 3d). Bees with more intense *N. ceranae* infections, as evidenced by higher numbers of spores in their ventriculi, tended to have lower levels of PPO and, potentially, PO.

4. Discussion

The experiment showed that the age of an individual when exposed to a parasite can have a significant effect on its survival, immunocompetence and the intensity of infections. Older honey bees developed more intense infections than younger bees when exposed to the *N. ceranae* parasite, and had lower levels of PPO, the immune enzyme precursor, with PPO levels overall correlating negatively with the intensity of parasite infections. However, older bees also survived infections better than younger bees. The experimental bees tested varied from 1 to 14 days of age at the time of exposure, and the effects of age are therefore likely to be even greater if the full age range of bees was compared, including overwintering bees and queens.

Individuals who were exposed to the parasite at an older age developed a more intense infection, having significantly more *N. ceranae* spores in their guts than younger bees. The greater infection intensity in older individuals may have consequences for the population-level transmission dynamics. Parasite loads in natural host populations often show an aggregation, with a minority of hosts producing a majority of parasite transmission (Woolhouse et al., 1997). This heterogeneity in transmission has been

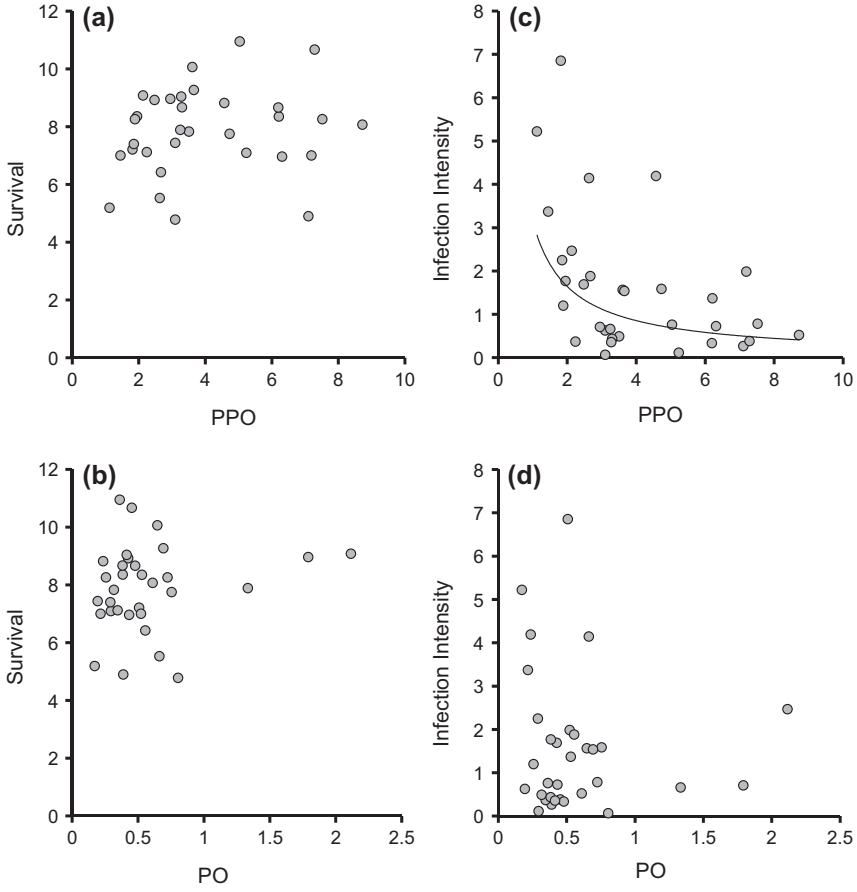

Fig. 3. Relationships across groups of bees, between immune enzyme levels and the survival of parasite exposed bees (a and b), and the intensity of *N. ceranae* infections (c and d). Enzyme activity is the V_{max}, change in absorbance over time during the linear phase of the enzymatic reaction of PPO (top graphs) and PO (bottom graphs), with mean survival time (days; left graphs) or the intensity of *N. ceranae* infections ($\times 10^6$ spores; right graphs). Trend line shown for significant correlation of PO and infection intensity ($y = -2 \times 10^6(x) + 4 \times 10^6$, $R^2 = 0.28$).

suggested to be due to encounter rates with parasites. However, our results demonstrate that heterogeneity in the production of parasite transmission propagules can also be due to physiological differences caused by the age of individuals.

Age differences in resistance to parasites have been suggested to be most likely due to immune function changes or immunosenescence (Amdam et al., 2005). Although immune enzyme levels in our study were highly variable, levels of PPO were reduced by exposure to the *N. ceranae* parasite to a greater extent in older bees. This suggests that older bees were either activating their immune system to a greater extent than younger bees in response to parasite challenge, or were less able to replace the activated PPO. The decline in PPO levels in the control bees may also be due to a decline with increasing age of the bees, or the stress associated with experimental handling and being maintained outside of the colony environment. Immunosenescence has been found previously in honey bees (including in PO activity), but only using bees collected directly from colonies, and therefore not from a controlled social environment (Laughton et al., 2011). Our results show that there is increased susceptibility to parasite infection with increasing age in bees, without the confounding influence of social context or task. As age increased so did infection intensity, in agreement with other evidence of immunosenescence in invertebrates (Adamo et al., 2001).

The negative correlation between PPO levels and the intensity of the *N. ceranae* infections suggests that the phenoloxidase component of the immune system in honeybees is impacted by infections with *N. ceranae*. *Nosema ceranae* has only recently emerged as a parasite of the European honey bee and bumblebees, and has received much interest due to its link with colony declines (Higes et al., 2007, 2008; Forsgren and Fries, 2010; Paxton, 2010; Graystock et al., 2013a,b; Furst et al., 2014). Studies investigating the ability of honey bees to resist *N. ceranae* infection have produced mixed results (Kralj and Fuchs, 2010; Paxton et al., 2008; Antúnez et al., 2009; Alaux et al., 2010; Forsgren and Fries, 2010; Suwannapong et al., 2011; Chaimanee et al., 2012; Dussaubat et al., 2012), but in many cases have either not controlled for age or used a range of host ages. Immune genes have been found to be down-regulated or up-regulated after infection with *N. ceranae* (Antúnez et al., 2009; Dussaubat et al., 2012), and phenoloxidase was found to not be affected by mixed *Nosema* species infections unless a pesticide stressor was also present (Alaux et al., 2010). This may also be the case in our results, where there was no effect on PO until several days after parasite exposure, when the stress of maintaining an immune response over an extended period may have become apparent. Previous work has suggested a role for PO in the immune response of invertebrate guts with haemolymph levels of the enzyme being strongly correlated with PO activity in both the cuticle and gut (Cotter and Wilson, 2002; Brown et al., 2003). Our findings show reduced levels of the immune precursor PPO, in parasite-exposed individuals, which correlates with greater infection intensity. This suggests that honey bees may be able to launch an immune response to *N. ceranae* infection, but highlight the importance of host age for drawing conclusions about parasite effects.

Our results demonstrate that host age in an insect can correlate with immune function and parasite resistance. Such age

differences may well in part explain the variation in virulence reported by studies with *N. ceranae* (Higes et al., 2007; Paxton et al., 2008; Forsgren and Fries, 2010; Graystock et al., 2013a; Furst et al., 2014), as well as other parasites. The importance of age will likely be even greater in the full age range of bees, including overwintering bees which live for many months longer than bees active during the summer, and queen bees which live for years compared to the weeks/months of workers. The results also highlight the highly dynamic nature of immune enzyme levels, and the limitations in general of using immune measures, such as PO and PPO, as proxies for potential host resistance to parasites (Adamo, 2004b,a). Immunosenescence can therefore occur in social insects independent of social context and task, and future studies will need to be consistent with host age for comparisons of parasite virulence, host survival and immune measures to be informative.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2014.06.004.

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