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Sex differences in immunity and rapid upregulation of immune defence during parental care in the burying beetle, *Nicrophorus orbicollis*

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Summary

1. Immunity may trade-off against other important life history traits, with recent work suggesting that reproduction and parental care in particular impinge on immune defence. However, whereas the effect of parental care on immunocompetence has been intensively studied in birds and mammals, virtually nothing is known about how it affects insect immunity.

2. Burying beetles provide extensive biparental care that includes the burial, preparation and defence of a carcass, as well as the subsequent feeding of the larvae. In addition, they cover the carcass with anal exudates that have been shown to serve an antimicrobial function (social immunity sensu *Behavioral Ecology*, **21**, 663–668). We examined the effect of sex, mating and parental care on measurements of individual and social immunity in the burying beetle *Nicrophorus orbicollis*.

3. Both males and females showed a rapid upregulation of the encapsulation response upon discovery of a carcass. The high encapsulation rate was maintained during the entire period of parental care. Lytic activity in anal exudates, a measure of social immunity, likewise increased. Mating had no effect on individual or social immunity, but females generally exhibited higher individual immunity than male *N. orbicollis*.

4. Our results suggest that the unusual breeding environment of burying beetles – a microbe-rich carcass – has selected for an atypical pattern of immune defence, with a significant upregulation of individual and social immunity during the physically demanding period of reproduction and parental care. The simultaneous investment in two life history traits that normally compete for resources may be an adaptive response in species that breed in environments with high densities of micro-organisms.

Key-words: burying beetles, encapsulation, immunity, life history trade-offs, lytic activity, parental care, phenoloxidase

Introduction

Immunity is an important component of fitness, and the elucidation of the selective forces shaping and maintaining immune defence is one of the recent major foci of evolutionary ecologists (e.g. Rolff & Siva-Jothy 2003; Schmid-Hempel 2005; Siva-Jothy, Moret & Rolff 2005; Stoehr & Kokko 2006; Nunn *et al*. 2009; Zuk 2009; Restif & Amos 2010). Models of the evolution of disease resistance are frequently grounded in life history theory (Roff 1992), which assumes that immune defence is a costly trait and, owing to finite resources, must be traded off against other life history traits such as reproduction (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Zuk & Stoehr 2002). The cost of reproduction and its implications for immunity are a central research issue, as exemplified by the numerous studies on birds and mammals that have revealed reduced immunocompetence and increased parasite susceptibility of parents during breeding (e.g. Festa-Bianchet 1989; Gustafsson *et al*., 1994; Norris, Anwar & Read 1994; Ots & Horak 1996; Deerenberg *et al*. 1997; Hanssen, Folstad & Erikstad 2003). Nearly every aspect of reproduction, including

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mating (e.g. Rolff & Siva-Jothy 2002; Leman *et al*. 2009; see also references in Lawniczak *et al*. 2007), oviposition (e.g. Siva-Jothy, Tsubaki & Hooper 1998) and parental care (e.g. Richner, Christe & Oppliger 1995; Deerenberg *et al*. 1997), has been shown to compromise immunity. However, although the effects of mating and oviposition on immunity have been investigated in insects, relatively little is known about the effects of parental care. In burying beetles, insects that reproduce on small dead vertebrates that serve as the sole food source for their offspring, males and females exhibit elaborate parental care (Pukowski 1933; Eggert & Müller 1997; Scott 1998). Parents are known to invest a considerable amount of time and energy in their young, which includes the defence, burial and preparation of the carcass (removing fur or feathers), as well as feeding of the larvae. Another unusual aspect of the life history of burying beetles is the fact that both females and males invest in 'social immunity' during parental care (Cotter & Kilner 2010b; Cotter *et al*. 2010). The term social immunity, in contrast to individual immunity, has been used to describe externally produced immune defences beneficial to offspring (Cotter & Kilner 2010a). For example, bark beetles coat the inside of the galleries in which they lay their eggs with oral secretions to protect the eggs against detrimental fungi (Cardoza, Klepzig & Raffa 2006). Burying beetles, on the other hand, are known to cover the carcass with oral and anal secretions, thereby inhibiting the growth of bacteria and other micro-organisms (Hoback *et al*. 2004; Jacques *et al*. 2009; Cotter & Kilner 2010b). This investment in resource defence is beneficial to their offspring, as competition with microbes has detrimental effects on larval growth (Rozen, Engelmoer & Smiseth 2008). Cotter *et al*. (2010) demonstrated that the parental investment in social immunity involves survival costs, but it is unknown whether this negative effect on longevity results from a trade-off between social and individual immunity. In general, the different investment strategies in individual vs. social immunity have been largely underexplored.

Although the physiological mechanisms underlying immune suppression may be diverse, studies have frequently attributed the suppressive effects to hormones. In vertebrates, testosterone has been shown to be a proximate explanation of the commonly found sex differences in immunity, with males frequently exhibiting a weaker immune response (Muehlenbein & Bribiescas 2005). In invertebrates, juvenile hormone (JH) has been shown to downregulate immune function in both sexes, and this trade-off is thought to be highly conserved in insects (Rolff & Siva-Jothy 2002). Given that female burying beetles undergo drastic hormonal (JH) changes during a reproductive bout (Trumbo, Borst & Robinson 1995; Scott *et al*. 2001; Panaitof, Scott & Borst 2004; Trumbo & Robinson 2008), burying beetles are an ideal model organism with which to examine potential trade-offs between hormone titre and immune defence.

In addition to the increased focus on the proximate mechanisms underlying immune suppression, the sexual dimorphism in immunocompetence often observed across various taxa has also received considerable attention. At an ultimate level, this difference has been generally attributed to differential selection on the sexes favouring different investment levels in parasite resistance. It is generally held that females gain more by investing in longevity, whereas male fitness is maximised by higher allocation to mating effort while sacrificing immune defence (Rolff 2002; Zuk & Stoehr 2002). In many vertebrates, males are indeed more susceptible to parasite infection, but in invertebrates, the picture is not so clear-cut and a meta-analysis has revealed no general differences in prevalence or intensity of parasite infections between the sexes (Sheridan *et al*. 2000). With respect to burying beetles, Cotter & Kilner (2010a) predicted that males should exhibit a stronger individual immune response than females. Male burying beetles typically desert the brood earlier than females, which has been explained by the finding that males have a greater residual reproductive value after a first breeding event than females (Ward, Cotter & Kilner 2009). Because of their greater residual reproductive value, males should gain more fitness from increased longevity than females.

In the current study, we examine a possible trade-off between immunity and parental care in insects, using the burying beetle *Nicrophorus orbicollis* as a model organism (Fig. 1). Specifically, we (i) determine whether there are any sex-specific differences in the allocation of resources to immune defence, (ii) investigate the effect of mating and parental care on measurements of both social and individual immunity and examine potential trade-offs between the two different forms of immunity and (iii) determine whether JH has an antagonistic effect on immunity in burying beetles by analysing immune responses at different times during parental care which correspond to different JH levels in females. For this examination, *N. orbicollis* is particularly suitable, as it is the only burying beetle species for which the entire JH profile during an entire breeding event is known (Trumbo 1997; Scott *et al*. 2001; Panaitof, Scott & Borst 2004).

Fig. 1. Female *Nicrophorus orbicollis* preparing a carcass during the period of parental care. Photograph taken by C. Köntgen.

Materials and methods

LIFE HISTORY, ORIGIN AND MAINTENANCE OF EXPERIMENTAL BEETLES

Male and female burying beetles provide elaborate parental care (Pukowski 1933; Eggert & Müller 1997; Scott 1998). They fly in search of small vertebrate carcasses that they bury within a few hours of discovery. Parental beetles remove the fur or feathers from the carcass, roll it into a ball and cover its surface in anal secretions, which have been shown to retard decomposition (Cotter & Kilner 2010b). In the North American species *N. orbicollis*, females begin oviposition about 15–36 h after the carcass has been discovered (see e.g. Panaitof, Scott & Borst 2004). Larvae hatch 3–4 days later and are fed regurgitated, predigested carrion by both parents. Females typically remain on the carcass longer than males, usually until larval development is complete about 6–8 days after hatching (Scott 1989, 1990; Trumbo 1991).

Experimental *N. orbicollis* were the first-generation offspring of beetles (n = 50 pairs) collected from carrion-baited pitfall traps established in the Merwin Nature Preserve, a tract of secondary deciduous forest bordering the Mackinaw River in McLean County, Illinois, USA (40°40′N, 88°53′W). To avoid any potential density effects on immunity, adult beetles were housed singly prior to being used in an experiment. From eclosion to the start of the experiment, beetles were kept in small plastic containers (480 ml) two-thirds filled with moist peat at 20 °C under a 16 L:8 D light regime and fed small pieces of ground beef twice a week.

GENERAL EXPERIMENTAL DESIGN

To determine the effect of sex, mating and parental investment on different aspects of immunocompetence, 169 male and 168 female *N. orbicollis* were randomly assigned to one of the five treatment groups (see Table 1 for overview). In the first treatment group (S = Single), virgin males and females were housed singly, and in the second group (P = Paired), beetles were kept in pairs (one male, one female). We did not observe how often the pairs copulated, but studies of other *Nicrophorus* species have documented repeated mating with the same partner (House *et al.* 2008, 2009; Steiger *et al.* 2008). In one video study of *Nicrophorus vespilloides*, pairs mated an average of 29 times in 24 h (Müller & Eggert 1989). The beetles of these non-breeding treatment groups (S and P) were provided with small pieces of ground beef *ad libitum* (i.e. two pieces per individual every other day) to ensure that they were well nourished.

In the remaining three ('breeding') treatment groups, monogamous pairs each were provided with a newly defrosted mouse carcass, *c.* 25 g in mass. To simulate postinterment conditions, all breeding beetles were held in the dark. In the third treatment group (BE = Breeding and Egg-laying), measurements of immune function (implant insertion, see description later) were initiated 24 h after carcass contact, the time at which females oviposit and both beetles prepare the carcass. After implant insertion, we promptly transferred pairs and the carcass to a new box and searched the old box for eggs. The new box was also searched for eggs 14 h later, after which beetles were sacrificed for implant recovery. Pairs in the fourth and the fifth treatment group were transferred to a new box 3 days after their first carcass contact. The old container was searched for eggs, which were transferred to a moist filter paper placed in a Petri dish. The Petri dishes were checked for the presence of newly hatched larvae three times a day. We provided each reproducing pair with a brood of 10 newly hatched larvae of mixed maternity. Because females kill larvae arriving before the hatching of their own brood (Müller & Eggert 1990), we provided females with broods only after their own larvae had started hatching. Pairs of the fourth treatment group (BL = Breeding and Larvae) were subjected to implantation after they had cared for larvae for 24 h (*c.* 6 days after carcass discovery), at which time both the rate of larval feeding and the JH III level in haemolymph are peaking (Fetherston, Scott & Traniello 1994; Scott *et al.* 2001; Smiseth, Darwell & Moore 2003; Panaitof, Scott & Borst 2004). After implantation, pairs were returned to their respective broods for 14 h. Pairs of the fifth and last treatment group (AB = After Breeding) did not receive an implant until the end of the breeding period, at which time the larvae begin to disperse from the carcass into the surrounding soil and JH III levels return to prebreeding values (*c.* 10–12 days after carcass discovery). After these beetles had been implanted, they were transferred to a fresh box without their dispersing larvae before being sacrificed for implant recovery.

We avoided possible confounding effects of handling differences between breeding and non-breeding groups by treating the non-breeding groups similar to breeding ones, transferring them to new boxes whenever the breeding beetles were transferred. Beetles of groups S and P were subjected to implantation at the same time as the individuals of group AB. Hence, after the start of the experiment, beetles of groups S and P were kept in boxes for a period of 10–12 days and were implanted at the same age as beetles of group AB.

Table 1. Overview of the different treatment groups used in the study

Groups	Status	Description
S	Non-breeding	Males and females were housed singly and fed *ad libitum*; implantation was conducted at the same day as in group AB
P	Non-breeding	Beetles were housed with a partner of the opposite sex and fed *ad libitum*; implantation was conducted at the same day as in group AB
BE	Breeding	Pairs of beetles were provided with a carcass; implantation was conducted 24 h after carcass provisioning, which corresponds to the time of egg laying
BL	Breeding	Pairs of beetles were provided with a carcass; implantation was conducted 24 h after hatching of larvae, which corresponds to the time of intensive parental care and high JH titre in females
AB	Breeding	Pairs of beetles were provided with a carcass; implantation was conducted at the end of the breeding period, when larvae leave the carcass for pupation

S, single; P, paired; AB, after breeding; BL, breeding and larvae; BE, breeding and egg-laying; JH, juvenile hormone.

MEASUREMENTS OF IMMUNITY

Insects possess innate immunity, comprised of humoral and cellular components (Gillespie, Kanost & Trenczek 1997; Söderhäll & Cerenius 1998; Lawniczak *et al.* 2007). We examined both humoral and cellular responses in our study with methods commonly used to assess insect immunity (see references in Gershman *et al.* 2010b). For each

experimental beetle, we made three measurements of individual immunity (i.e. encapsulation ability, phenoloxidase (PO) activity and lytic activity in haemolymph) and two measurements of social immunity (i.e. PO activity and lytic activity in anal secretion). This enabled us to analyse the correlation between social and individual immune response and also between different measures of immunity. Although PO is typically used as a measure of individual immune function because it is part of the encapsulation response occurring in the haemolymph, we also measured its activity in the anal secretion because PO also generates toxic quinones and other reactive components contributing to the killing of pathogens and parasites (Gillespie, Kanost & Trenczek 1997).

Encapsulation rate

To measure encapsulation rate, all beetles were implanted with a 3-mm long, sandpaper-roughened segment of 0·255-diameter nylon monofilament fishing line. A small hole was made between the fourth and fifth abdominal sternites with a 27-gauge syringe needle, and the implant was inserted until it was completely contained within the beetle's abdominal cavity. Prior to use, the needle and implants were sterilised in 70% ethanol. After implantation, beetles were returned to their respective containers and allowed to resume their activity. A pilot study using 10 males and 10 females revealed that 14 h was an appropriate duration for the implants to remain in the beetles to provide a range of variation in melanisation. Exactly 14 h after receiving an implant, beetles were freeze-killed and stored in a −80 °C freezer.

We dissected the frozen beetles to recover implants, removing any clumps of tissue adhering to the implants. Each implant was photographed three times from different angles next to a clean implant control using a Nikon Coolpix 4500 digital camera mounted on a stereomicroscope (Wild Heerbrugg Ltd, Heerbrugg, Switzerland). We measured the degree of implant melanisation using ImageJ image-analysis software freely available from the National Institutes of Health (http://rsbweb.nih.gov). We outlined each implant and control using the polygon tool, which produced an average greyscale value from all the pixels within each image. Darkness scores range from 0 (completely white) to 256 (completely black). The darkness score for each individual was calculated as the average difference between implant and control scores in the three images analysed.

Phenoloxidase and lytic activities

Shortly before freeze-killing an experimental beetle, we used a capillary tube to collect 1·5 μl anal exudate and 3 μl haemolymph. Anal exudate can be obtained by handling the beetles, which usually causes them to release the brown liquid from the anus (Cotter & Kilner 2010b). We mixed haemolymph with 40 μl and anal exudate with 20 μl of phosphate-buffered saline (PBS) and stored the samples in a −80 °C freezer until further processing.

To estimate PO activity, we added a known quantity of L-3,4-dihydroxyphenylanine (L-DOPA) to the haemolymph and anal exudate to replace the naturally occurring substrate. Because the amount of L-DOPA was constant across samples, any resulting differences in melanin production were owing to individual differences in PO activity. We combined 5 μl of thawed haemolymph or anal exudates solution with 7 μl of bovine pancreas α-chymotrypsin (Sigma-Aldrich, Steinheim, Germany) in each well of a spectrophotometer microplate and incubated the mixture for 20 min at room temperature (20 °C). α-Chymotrypsin acts as a catalyst and converts all PPO present in the solution into PO (Bailey & Zuk 2008). We then added 90 μl of 15 mM L-Dopa (Sigma-Aldrich) to each well and recorded optical density (OD) at 490 nm using a Power Wave 340 Microplate Spectrophotometer with Kc4 data analysis software (Bio-Tek Instruments, Richmond, Virginia, USA). This method estimates the total change in OD over the course of the reaction, ranging from an OD of 0 (transparent) to 4 (opaque). OD readings were taken every 10 min for 210 min. The PO activity rate was calculated as the change in OD over time (OD/time). We performed the same calculation on 12 control wells of each 96-sample plate containing only PBS and L-DOPA and then subtracted the average value of control samples from individual beetle values to obtain a final PO level. These protocols were adapted from Gershman *et al.* (2010a,b).

To determine the ability of lysozyme-like enzymes to induce bacterial cell lysis, we used 3 mg of *Micrococcus lysodeikticus* (Sigma-Aldrich), a Gram-positive bacterium, per 10 ml of PBS buffer. To estimate lytic activity, 10 μl of thawed haemolymph or anal exudate sample, along with 90 μl of PBS/*M. lysodeikticus* solution, was added to each spectrophotometer microplate well and changes in OD recorded. This method estimates the total change in OD from opaque to clear as lysozymes or lysozyme-like enzymes lyse the bacterial cells. OD readings were recorded every 5 min for 150 min. We performed the same calculation on 12 control wells of each 96-sample plate containing only PBS/*M. lysodeikticus* solution and then subtracted the average value of control samples from individual beetle values to obtain a final level of lytic activity. Although OD decreases as more bacterial cells are lysed, lytic activity is given as a positive number for clarity. The experimental design described does not allow the characterisation of the specific lysozyme responsible for cell lysis; the observed lytic activity is thus attributed to a lysozyme-like enzyme.

STATISTICS

All statistical analyses were performed using SPSS 18 (SPSS Inc., Chicago, Illinois, USA). A multivariate linear mixed effects REML model was used to assess the effect of sex and treatment on components of immunity. Pair ID was included as a random effect. In the treatment in which the beetles were held without a partner (treatment group S), each individual received its own unique ID. As we were not able to test all beetles simultaneously, our sample contained two distinct age groups: adults were either 22 or 38 days old at the beginning of the experiment. We added age as a factor in the mixed model and included body size (measured as pronotum width) as a covariate. We performed two separate mixed models: one which analysed the different components of individual immunity (i.e. implant darkness, PO activity and lytic activity in haemolymph) and a second which analysed the two components of social immunity (PO activity and lytic activity in anal exudates). Follow-up contrasts were evaluated using the sequential Bonferroni method.

To examine the relationship between different components of immunity, we calculated pairwise Pearson correlations between the individual measures (i.e. implant darkness, PO and lytic activities in haemolymph and anal exudate). The increased error rate owing to multiple pairwise comparisons was corrected by controlling the false discovery rate (Benjamini & Hochberg 1995).

Overall, we were able to obtain at least one measurement of immunity from 316 of 337 experimental beetles. Beetles that died before the end of the experiment, did not lay fertilised eggs, or killed their brood were excluded from the analysis.

Results

INDIVIDUAL IMMUNITY

Both sex and treatment had significant effects on measures of individual immunity (sex: $F_{1,\ 837} = 7{\cdot}39$, $P < 0{\cdot}01$; treatment: $F_{4,136} = 15{\cdot}10$, $P < 0{\cdot}001$). There was a significant interaction between treatment and age ($F_{4,\ 136} = 2{\cdot}81$, $P < 0{\cdot}01$) in their effects on individual immunity. Later, we use follow-up univariate linear mixed effects REML models for the different measures of individual immunity to evaluate their contribution to the significant multivariate linear mixed model effects (Table 2).

Encapsulation rate

Encapsulation rate depended significantly on both sex and treatment (Table 2; Fig. 2). Females showed a generally higher encapsulation rate than males. There was no significant interaction between sex and treatment or between sex and age (Table 2). Regarding the effect of treatment, pairwise comparisons revealed that males and females of all three groups tested when reproducing on a carcass (BE, BL and AB) had significantly darker implants than the beetles of the two non-breeding groups (S and P) (Table 3, Fig. 2). There was no difference in implant darkness between the different groups of breeding beetles (Table 3, Fig. 2), and thus, encapsulation rate apparently did not depend on the amount of time the beetles had spent on a carcass. Mating also did not affect implant darkness, as there was no difference between beetles kept alone (S) and those held with a mating partner (P) (Table 3, Fig. 2). Age had no effect on encapsulation rate; there was, however, a significant interaction between age and treatment on implant darkness (Table 2), with older beetles showing a slightly higher encapsulation rate than younger ones when not breeding (S, P), but a slightly lower encapsulation rate than younger ones when breeding (BE, BL and AB).

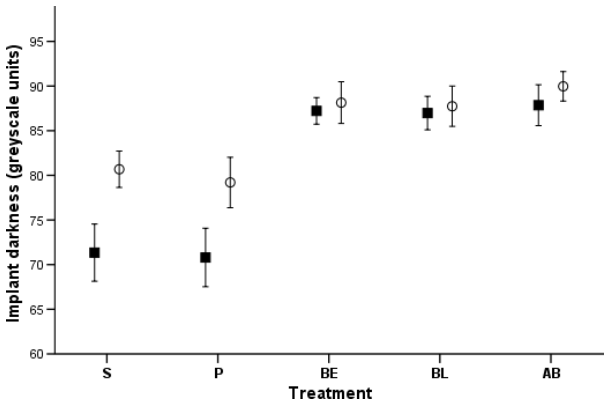

Fig. 2. Implant darkness (mean ± SE) of males (black squares) and females (open circles) from five different treatment groups. S, single non-breeding beetles; P, paired non-breeding beetles; BE, breeding beetles during time of egg laying; BL, breeding beetles during intensive period of larvae care; AB, beetles shortly after breeding period, when larvae leave the carcass for pupation. Results of the statistical analyses are reported in Tables 2 and 3.

Table 2. Follow-up univariate linear mixed effects REML model of the fixed effects of sex, treatment, age and their interactions on measurements of individual immunity (implant darkness, PO and lytic activities in haemolymph). Pair ID was included as random effect and size was included as a covariate. Bold values are statistically significant

Factors	Implant darkness	PO activity (haemolymph)	Lytic activity (haemolymph)
Sex	**$F_{1,154} = 9{\cdot}99$** **$P = 0{\cdot}002$**	**$F_{1,157} = 4{\cdot}73$** **$P = 0{\cdot}031$**	$F_{1,140} = 0{\cdot}42$ $P = 0{\cdot}52$
Treatment	**$F_{4,147} = 14{\cdot}50$** **$P < 0{\cdot}001$**	$F_{4,156} = 1{\cdot}13$ $P = 0{\cdot}34$	$F_{4,136} = 0{\cdot}74$ $P = 0{\cdot}57$
Age	$F_{1,168} = 3{\cdot}57$ $P = 0{\cdot}06$	$F_{1,181} = 2{\cdot}07$ $P = 0{\cdot}15$	$F_{1,164} = 0{\cdot}28$ $P = 0{\cdot}60$
Sex*treatment	$F_{4,128} = 2{\cdot}34$ $P = 0{\cdot}058$	$F_{4,147} = 0{\cdot}54$ $P = 0{\cdot}71$	$F_{4,134} = 1{\cdot}26$ $P = 0{\cdot}29$
Sex*age	$F_{1,151} = 0{\cdot}874$ $P = 0{\cdot}35$	$F_{1,155} = 0{\cdot}32$ $P = 0{\cdot}57$	$F_{1,138} = 14$ $P = 0{\cdot}71$
Treatment*age	**$F_{4,146} = 3{\cdot}14$** **$P = 0{\cdot}016$**	$F_{4,156} = 0{\cdot}52$ $P = 0{\cdot}72$	$F_{4,137} = 1{\cdot}34$ $P = 0{\cdot}26$
Sex*treatment* age	$F_{4,128} = 1{\cdot}41$ $P = 0.24$	$F_{4,257} = 2{\cdot}96$ $P = 0{\cdot}06$	**$F_{4,135} = 3{\cdot}12$** **$P = 0{\cdot}02$**
Size	$F_{1,283} = 3{\cdot}47$ $P = 0{\cdot}06$	$F_{1,269} = 2{\cdot}54$ $P = 0{\cdot}11$	$F_{1,264} = 0{\cdot}53$ $P = 0{\cdot}47$

Table 3. Pairwise comparisons assessing the differences between the five treatment groups in implant darkness and lytic activity in anal exudates. Adjustment for multiple comparisons was made by performing a Bonferroni correction. Bold values are statistically significant

		P	
Treatment (I)	Treatment (J)	Implant darkness	Lytic activity (anal exudates)
S	P	1·00	1·00
	BE	**<0·001**	0·35
	BL	**<0·001**	**0·01**
	AB	**<0·001**	**0·02**
P	BE	**<0·001**	0·55
	BL	**<0·001**	**0·02**
	AB	**<0·001**	**0·03**
BE	BL	1·00	1·00
	AB	1·00	1·00
BL	AB	1·00	1·00

S, single; P, paired; AB, after breeding; BL, breeding and larvae; BE, breeding and egg-laying.

Phenoloxidase and lytic activities in haemolymph

Sex had an effect on the PO activity in haemolymph, with females exhibiting higher values than males (Table 2; Fig. 3a). No further effect on PO activity could be detected. Lytic activity in the haemolymph was not affected by sex, treatment or age (Table 2, Fig. 3b). However, there was a significant interaction involving all three factors (Table 4).

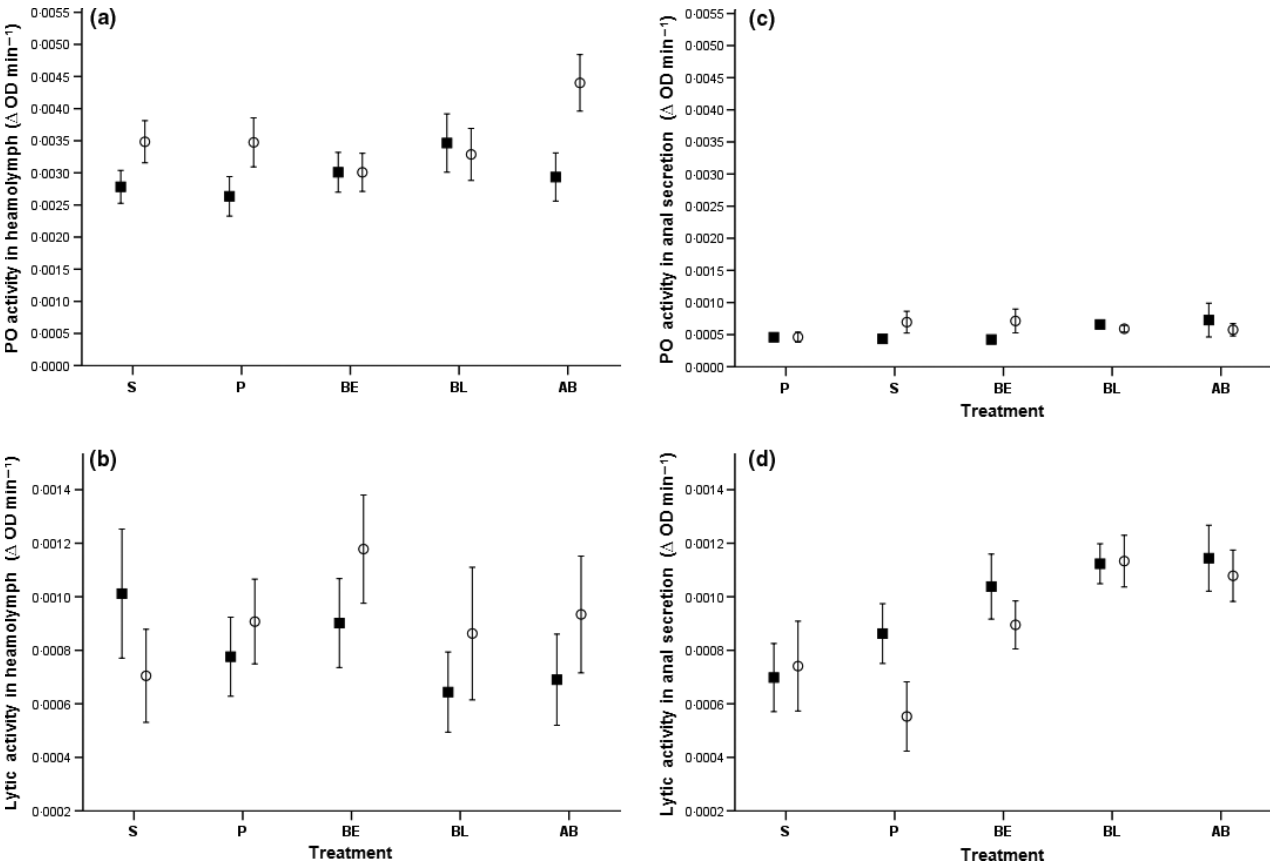

Fig. 3. Individual immunity (a, b) and social immunity (c, d) of males (black squares) and females (open circles) from five different treatment groups. S, single non-breeding beetles; P, paired non-breeding beetles, BE, breeding beetles during time of egg laying; BL, breeding beetles during intensive period of larvae care; AB, beetles shortly after breeding period, when larvae leave the carcass for pupation. (a) PO activity in haemolymph (mean ± SE), (b) lytic activity in haemolymph (mean ± SE), (c) PO activity in anal exudates (mean ± SE), (d) lytic activity in anal exudates. Enzyme activity was measured as change in optical density (OD) over time was measured. Results of the statistical analyses are reported in Tables 3 and 4.

Table 4. Follow-up univariate linear mixed effects REML model of the fixed effects of sex, treatment and age on measurements of social immunity (PO and lytic activities in anal exudates). Pair ID was included as random effect, and size was included as a covariate. Interactions are not shown as there was no significant interaction between their effects. Bold values are statistically significant

Factors	PO activity (anal exudates)	Lytic activity (anal exudates)
Sex	$F_{1,205} = 2{\cdot}99$ $P = 0{\cdot}09$	$F_{1,205} = 2{\cdot}31$ $P = 0{\cdot}13$
Treatment	$F_{4,556} = 0{\cdot}46$ $P = 0{\cdot}76$	$\mathbf{F_{4,559} = 5{\cdot}29}$ $\mathbf{P < 0{\cdot}001}$
Age	$F_{1,518} = 0{\cdot}83$ $P = 0{\cdot}36$	$F_{1,524} = 0{\cdot}32$ $P = 0{\cdot}57$
Size	$F_{1,290} = 0{\cdot}23$ $P = 0{\cdot}63$	$F_{1,293} = 0{\cdot}23$ $P = 0{\cdot}63$

SOCIAL IMMUNITY

Phenoloxidase and lytic activities in anal exudates

Phenoloxidase activity in anal exudate samples was lower than in haemolymph samples ($F_{1,451} = 406{\cdot}28$, $P < 0{\cdot}0001$, see Fig. 3a,c), whereas no difference could be detected in the lytic activity of the two types of samples ($F_{1,571} = 0{\cdot}60$, $P = 0{\cdot}44$, see Fig. 3b,d).

Neither sex nor age had a significant effect on immunity measures in exudate samples (sex: $F_{1,1386} = 0{\cdot}014$, $P = 0{\cdot}91$, age: $F_{1,135} = 1{\cdot}16$, $P = 0{\cdot}28$), but there was a significant treatment effect ($F_{4,124} = 3{\cdot}88$, $P = 0{\cdot}005$). Follow-up univariate mixed models revealed that in anal exudate, treatment affected lytic activity, but not PO activity (Table 4; Fig. 3c,d). Pairwise comparisons revealed that the groups in which beetles had fed larvae (BL, AB) exhibited higher lytic activity in their anal exudate than beetles of the other two groups (S, P and BE; Table 3, Fig. 3d).

RELATIONSHIP BETWEEN DIFFERENT COMPONENTS OF IMMUNITY

There was no evidence of the predicted trade-off, or negative correlation, between components of individual and social immunity. Instead, we found a positive correlation: implant darkness correlated positively with lytic activity in anal exudate (Pearson correlation, $n = 226$, $r = 0{\cdot}19$, $P = 0{\cdot}004$).

Between the different measurements of individual immunity, however, there was a negative correlation: PO activity and lytic activity in haemolymph were negatively correlated (Pearson correlation, $n = 287, r = -0{\cdot}19, P = 0{\cdot}002$).

OFFSPRING NUMBER AND IMMUNITY

Females that had been breeding on a carcass and completed oviposition (BL, AB) laid an average of 40 (SD = 11; $n = 62$) eggs. There was no relationship between clutch size and any of the immunity measures (pairwise Pearson correlations, all $P > 0{\cdot}1$). Although all beetles were initially provided with 10 first instar larvae, there was some variation in how many offspring (AB) survived (mean ± SD: 8 ± 2; $n = 33$). However, there was no correlation between offspring number and any of the immunity measures (pairwise Pearson correlations, all $P > 0{\cdot}1$).

Discussion

Our study of immunity in the burying beetle *N. orbicollis* yielded three main results: (i) pairs reproducing on a carcass showed equal or greater measures of individual immunocompetence than non-breeding beetles (higher encapsulation rate, equal PO and lytic activities in haemolymph), (ii) across treatments, males had a lower encapsulation rate and PO activity in haemolymph than females and (iii) the antibacterial (lytic) activity of the anal secretion that beetles release when disturbed, and that they also spread on a carcass they have buried, was elevated during reproduction on a carcass by both males and females.

The increase in the encapsulation response during breeding and parental care required the presence of a carcass and was not triggered by sexual activity or the availability of a mating partner alone. It could be argued that the increased encapsulation in breeding beetles was a direct response to the presence of pathogens associated with the carcass, as insects typically increase their immune function after exposure to an immune challenge (Gillespie, Kanost & Trenczek 1997; Lemaitre, Reichhart & Hoffmann 1997; Söderhäll & Cerenius 1998; Haine *et al.* 2008; Cotter, Ward & Kilner 2011). However, the carcasses we used were clean and pathogen free at the beginning of the experiment, as they had been frozen for several weeks and were thawed in clean containers just prior to the experiment. The first immunity measurement was taken 24 h after the receipt of a carcass by a breeding pair, and immune function was already increased in comparison with non-breeding beetles. The immune challenge posed by bacteria, fungi or other organisms colonising the carcass should have increased over the initial period of a breeding attempt as these organisms became more abundant, and then decreased towards the end of breeding when the carcass was mostly consumed and micro-organisms, less abundant. Thus, we might have expected a decreased encapsulation response at the end of breeding, but instead, darkness scores were elevated evenly in all breeding groups.

If the increased encapsulation response on a carcass is not a direct response to infection, it might be interpreted as a preemptive measure against the higher risk of encountering pathogens during breeding. Encapsulation may aid the beetles in coping with bacteria, fungi or parasitic organisms that quickly colonise a carcass and may negatively affect the beetles' condition. However, the primary benefit associated with improved encapsulation may be a slightly different one. Non-breeding burying beetles normally do not fight with each other, but on carcasses suitable for reproduction, fights are numerous and often quite violent and can lead to the partial or complete loss of legs or antennae or even death (Pukowski 1933; Scott 1990; Trumbo 1990; Eggert & Müller 1992). Injuries sustained in fights often leave the beetles with lesions in their exoskeleton that need to be closed rapidly because they offer ready sites of entry for pathogens and increase the risk of dehydration because of the evaporation of haemolymph. Melanisation plays a key role in both insect wound healing and encapsulation (Gillespie, Kanost & Trenczek 1997), and improved melanisation of a foreign object is associated with improved closing of open wounds. Changes in the composition of the complex blend of cuticular hydrocarbons during breeding (Steiger *et al.* 2007; Steiger, Peschke & Müller 2008) may serve a similar function, namely shielding the beetle from external pathogens, especially fungi (Herzner & Strohm 2007). It must be acknowledged, however, that once the carcass has been mostly consumed, conflict over its possession is relaxed, and thus, the high encapsulation response of beetles observed at the end of breeding is puzzling. It may be that while individual immunity is rapidly upregulated in response to need, it is slow to downregulate when the need dissipates. Indeed, in experiments with bumble bees, individuals upregulated immune defence shortly after an immune challenge, but it decreased only slowly thereafter, so that even after 14 days, significant antibacterial activity could still be detected (Korner & Schmid-Hempel, 2004).

Apparently, burying beetles are able to increase their investment in individual immune defence at a time when they are also engaging in a number of other physiologically demanding activities. Breeding females have to complete ovarian development (vitellogenesis) very rapidly while burying and preparing the carcass, and after oviposition, they still have to maintain the carcass and regurgitate predigested food to the young. While males assist females in burying and preparing the carcass, they also mate very frequently (on average, 79 times in the first 48 h after carcass discovery: Müller & Eggert 1989), and later, they help feed and defend the young. Juvenile hormone titres in both sexes are elevated throughout a breeding attempt, but especially during parental feeding and defence of larvae (Trumbo 1997; Scott *et al.* 2001; Panaitof, Scott & Borst 2004). Increased JH concentration may function to increase the overall metabolism of breeding beetles, thus allowing for the maintenance of continuous activity during breeding (Trumbo & Robinson 2008).

The lack of a trade-off between immunity and reproduction may seem surprising considering the multitude of other studies that have documented such trade-offs in other systems.

Many avian studies, for example, have found evidence of reduced immune function during egg-laying and parental care (Gustafsson *et al*., 1994; Deerenberg *et al*. 1997; Hanssen, Folstad & Erikstad 2003). In most insects, high titres of JH are associated with reproduction (mating in males and oviposition in females), and reduced immunocompetence occurs during reproduction or when the concentration of JH in haemolymph is high (Rolff & Siva-Jothy 2002; Contreras-Garduno *et al*. 2009). However, there is a fundamental difference between burying beetles and many of these other systems that may be important in this context. Breeding beetles find themselves in the unusually fortunate situation of unfettered access to a prime source of protein-rich food, which may enable them to simultaneously invest in two otherwise competing functions (see, e.g., Lee, Simpson & Wilson 2008 for a positive effect of diet on immune function). Our result suggests that even trade-offs that appear to be extremely common can be mitigated by a combination of unusual selective pressures (greatly increased risk of pathogenesis during breeding) and opportunities (access to high-quality nutrition). However, it is possible that this double investment comes at the expense of future reproduction and survival. Both female life span and fecundity in subsequent reproductive bouts are reduced after just one breeding attempt when compared with non-breeding beetles (Creighton, Heflin & Belk 2009; Ward, Cotter & Kilner 2009).

Our finding of lower immunocompetence in males was not unexpected: the majority of studies in insects have revealed the same pattern (see metanalysis in Nunn *et al*. 2009), as have studies in vertebrate systems (Muehlenbein & Bribiescas 2005). Females exhibit superior immune function in several insect orders, e.g., Orthoptera (Gershman *et al*. 2010b), Odonata (Rolff 2001), Diptera (Schwarzenbach, Hosken & Ward 2005) and Mecoptera (Kurtz *et al*. 2000). Most authors interpret these findings within the framework of the different life history strategies of males and females and the greater benefits of increased life span to females compared with males. Cotter & Kilner (2010a) predicted, however, that in burying beetles, females should invest less effort than males into their individual immune defence because after one brood, female *N. vespilloides* produce fewer and smaller offspring in subsequent reproductive bouts, whereas males do not, leading to the conclusion that females gain less from increased longevity than males (Ward, Cotter & Kilner 2009; Cotter & Kilner 2010a). We suggest instead that it is equally reasonable to expect that the effects of longevity on lifetime fitness are stronger in females than in males and that females should therefore allocate more resources to immunity, as they did in the present study. The number of offspring a female burying beetle is able to produce depends predominantly on the number of carcasses she can discover in her life. Although carcasses are a rare and unpredictable resource (Eggert & Müller 1997), the number of carcasses a female can expect to find will increase with increased longevity and activity. Males, on the other hand, can sire young even if they never find a carcass, engaging in pheromone emission to attract females that they inseminate in the absence of a reproductive resource (Müller & Eggert 1987; Eggert & Müller 1989; Beeler, Rauter & Moore 1999). Pheromone-emitting males often are able to sire at least some offspring (Müller *et al*. 2007), particularly if females bury a carcass without the help of another male, which happens regularly (Eggert 1992; Müller *et al*. 2007; Scott, Lee & Van Der Reijden 2007). We do not know whether male pheromone emission increases their total daily activity when compared with females, but pheromone emission may entail additional costs in terms of pheromone production (see, e.g., Rantala, Vainikka & Kortet 2003) or increased predation risk (Sakaluk 1990). Thus, male burying beetles may gain less from increased longevity than females or sacrifice some immune function for mating effort, which may account for the consistent sex difference observed in our study. Females may also be subject to stronger selection for improved immune function, given that they can undertake a reproductive attempt by themselves (Eggert & Müller 1997; Scott 1998). Females also spend more time feeding larvae and processing carrion than males (Smiseth *et al*., 2005), which presumably would expose them to microbes to a greater extent.

The effectiveness of anal exudate in reducing bacterial growth was increased during breeding. Male and female burying beetles spread anal exudate on carcasses they have buried, which has often been suggested to function in the control of bacteria and fungi (Hoback *et al*. 2004; Rozen, Engelmoer & Smiseth 2008). Consistent with this suggestion and similar to the finding of Cotter & Kilner (2010b) in *N. vespilloides*, our study revealed that in anal exudate, lytic activity was upregulated following the discovery of a carcass, while PO activity was not. It may be that lysozyme is only enhanced in response to the presence of micro-organisms, which may be adaptive given that changes in lytic activity have been shown to reduce longevity in burying beetles (Cotter *et al*. 2010). Consistent with this hypothesis, the antibacterial activity of exudate increased when the number of bacteria on a carcass was experimentally augmented (Cotter *et al*. 2010). Male and female *N. orbicollis* did not differ in the antimicrobial activity of their anal secretion. In *N. vespilloides*, however, the lytic activity of exudate was found to be affected by sex, both in breeding and in non-breeding beetles, although the difference was obvious on only one of the 3 days when beetles were tested (Cotter & Kilner 2010b).

During undisturbed breeding attempts, we found no evidence of trade-offs between immunity measures in haemolymph and anal exudate (individual and social immunity *sensu* Cotter & Kilner 2010a). On the contrary, because both encapsulation rate in the haemolymph and lytic activity in exudate increased during breeding, the two measures were significantly and positively correlated in our study. However, we found a trade-off between two different measurements of immunity in haemolymph: PO activity was negatively correlated with lytic activity. The same trade-off has been observed in earlier studies (e.g. Rantala & Kortet 2003; Cotter, Kruuk & Wilson 2004), supporting the notion that different components of immune defence may compete for limited resources within an individual. It is thus not surprising that

not all measures of immunocompetence responded similarly to our treatments. Many studies have found a similar lack of congruence between different immunity measures (Fedorka & Zuk 2005; Gershman 2008; Leman *et al.* 2009). In our study, breeding affected encapsulation rate but not PO activity, although PO is a key enzyme in the biochemical cascade leading to encapsulation (Cerenius & Söderhäll 2004). However, PO is only one component in the complex encapsulation process, and our experimental procedure caused inactive pro-PO to be converted to PO (Adamo 2004), which may have obscured a possible effect of breeding on levels of active PO in haemolymph.

In the present study, we attempted to identify possible effects of a reproductive attempt on immunity, while holding brood size constant. Consequently, brood size varied little and, not surprisingly, we found no effect of the number of offspring raised on immunity. It may be, however, that had experimental beetles been forced to rear a greater number of larvae, a trade-off between immunity and reproduction might have been manifest. Brood size is easily manipulated in burying beetles, and female *N. orbicollis* caring for experimentally enlarged broods have been found to suffer survival and fecundity costs (Creighton, Heflin & Belk 2009). Similar brood size manipulations could also be used in burying beetles to examine the trade-off between immunity and parental effort, as they have been in many avian systems (e.g. Richner, Christe & Oppliger 1995; Nordling *et al.* 1998). Any interaction between immune effort and reproductive investment could also be revealed by simultaneously manipulating the severity of the immune challenge that breeding beetles experience on a carcass, or by exposing them to an immune challenge prior to a breeding bout.

Our study revealed an interaction between the effects of treatment and age on encapsulation rate. In non-breeding beetles, the encapsulation response was stronger in older beetles, but among breeders, older beetles had a weaker response than younger ones; in both age groups, breeding individuals exhibited higher immune function than non-breeders. This result may indicate age-dependent investment patterns. If increased investment in superior brood care can be achieved by reducing immunocompetence, older breeding beetles may sacrifice immune defence to allocate more resources to reproduction (Creighton, Heflin & Belk 2009), consistent with the terminal investment hypothesis (Williams 1966; Clutton-Brock 1984). Alternatively, adverse effects of ageing might force older beetles to invest more into immune function even when not breeding, which might make them unable to summon an optimal increase in encapsulation when they encounter a carcass. The immune system of older beetles might also be less adaptable, thus reducing the magnitude of their response to changing situations.

The upregulation of encapsulation in breeding beetles occurred rapidly and was evident 38 h after carcass discovery (implants were inserted 24 h after carcass provisioning and remained inside the abdominal cavity for 14 h). When burying beetles have discovered a carcass, they exhibit several rapid behavioural and physiological responses, presumably adaptations to the rapidly decreasing value of their breeding resource (Scott & Traniello 1987). Juvenile hormone is a central hormone in insect reproduction (Nijhout 1994), although its exact function in burying beetles remains unclear (see, e.g., Panaitof & Scott 2006). The observation that JH titres in haemolymph increase very quickly when beetles locate a carcass (10- to 15-fold in less than an hour; Trumbo & Robinson 2008) suggests that JH may play an important role in many of these rapid responses, including the upregulation of immunity. This result is in direct contrast to other studies showing downregulation of immune function in response to increased JH, suggesting that the physiological trade-off between JH and immunity is not as highly conserved as once thought (see also Schwarzenbach, Hosken & Ward 2005). That high individual and social immunity occurred during both a high-JH period (active care) and a low-JH period (end of care), suggests that the initial JH surge upon carcass discovery triggers the immune response (Cotter & Kilner 2010b) and that subsequent JH fluctuations during the parental period have little effect on immunity.

Whatever the underlying mechanism, our results illustrate that an adaptive situation-dependent upregulation of immune function in insects can be very rapid. Other studies that have documented such plasticity in vertebrate and invertebrate systems have usually found it to be much slower. In rodents and other mammals, immune functions are seasonally upregulated over the course of several weeks in late fall and early winter (Nelson & Demas 1996; Sinclair & Lochmiller 2000; Nelson 2004). Social insects may enhance immune function in response to the frequency of social contacts, a phenomenon known as density-dependent prophylaxis (DDP) (Wilson *et al.* 2002), which takes about 8 days to become evident in bumblebee workers (Ruiz-Gonzalez, Moret & Brown 2009).

In conclusion, our study demonstrates that immunity is a plastic trait that can be rapidly adjusted to the existing pathogen risk. The unusual breeding environment of burying beetles has selected for an atypical pattern of immune defence with a significant upregulation of immunity, both in haemolymph and in anal exudate, during reproduction and parental care. If this simultaneous double investment in reproduction and immunity evolved owing to the exceptionally high density of micro-organisms on carcasses, we might predict a similar pattern in other organisms, such as dung beetles (Halffter, 1997), that utilise similar microbe-rich, high-protein resources for reproduction.

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