

# Sexual division of antibacterial resource defence in breeding burying beetles, *Nicrophorus vespilloides*

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## Summary

1. A key component of parental care involves defending resources destined for offspring from a diverse array of potential interspecific competitors, such as social parasites, fungi and bacteria.
2. Just as with other aspects of parental care, such as offspring provisioning or brood defence, sexual conflict between parents may arise over how to share the costs of this form of care. There has been little previous work, however, to investigate how this particular burden might be shared.
3. Here, we describe a hitherto uncharacterized form of parental care in burying beetles *Nicrophorus vespilloides*, a species which prepares carrion for its young and faces competition from microbes for this resource. We found that parents defend the carcass with antibacterial anal exudates, and that the antibacterial activity of these exudates is only upregulated following the discovery of a corpse. At the same time, phenoloxidase activity in the anal exudates is downregulated, indicating parallels with the internal insect immune system.
4. In unmanipulated breeding pairs, females had higher antibacterial activity in their anal exudates than males, suggesting sex-specific roles in this aspect of parental care.
5. When we experimentally widowed males, we found that they increased levels of antibacterial activity in their anal exudates. Experimentally widowing females, however, led them to decrease levels of antibacterial activity in their anal exudates. Widowed beetles of each sex thus produced anal exudates of comparable antibacterial activity. We suggest that this flexible division of antibacterial activity may be coordinated by Juvenile Hormone.

**Key-words:** biparental care, lysozyme, partial compensation, phenotypic plasticity, social immunity

## Introduction

Parents rearing offspring commonly face competition from other species for the resources they wish to devote to their young. Sometimes these competitors intercept food as it is brought back to the nest (e.g. Taylor 1979; Stienen & Brenninkmeijer 1999), but often rivals position themselves within the nest to steal resources intended for offspring. The behaviours of juvenile social and brood parasites are particularly well known in this regard (e.g. Davies, Bourke & Brooke 1989; Davies 2000; Barbero *et al.* 2009), but fungi and other micro-organisms also pose a threat to developing young by competing for resources that are otherwise destined for offspring. The 'cuckoo' fungus invades termite nests, for example, where it steals the attention of the workers who groom the sclerotia and keep them free from pathogens instead of providing the same care to termite eggs (Matsuura 2006;

Matsuura *et al.* 2009). Fungi also threaten the development of spruce beetle larvae by invading the galleries within the host trees where they develop (Cardoza, Klepzig & Raffa 2006), while fungi and other micro-organisms compete with larval burying beetles for the resources on a small vertebrate carcass (Rozen, Engelmoer & Smiseth 2008).

Parents have evolved a range of counter-measures to reduce the extent of this competition. For example, hosts of both the social and brood parasites actively repel potential parasites (e.g. Turillazzi, Cervo & Cavallari 1990; Davies 2000; Rasa & Heg 2004; Welbergen & Davies 2009) and, should those defences fail, are also adept at recognizing and rejecting intruders in the nest (e.g. Davies 2000; Brandt *et al.* 2005; Matsuura 2006). Furthermore, there is increasing evidence that adults caring for dependent young may also have antimicrobial strategies at their disposal (West & Alexander 1963; Currie *et al.* 1999; Hart 2005; Rozen *et al.* 2008; Scott *et al.* 2008). For example, spruce beetles use antifungal oral secretions to minimize the threat

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to their young from competing fungi (Cardoza *et al.* 2006), while wood ants carry solidified coniferous resin back to their nests for use as both an antifungal and antibacterial agent (Chapuisat *et al.* 2007).

The defence of offspring resources against competitors in this way is thus a key component of parental care, and it has several features in common with the better characterized components of care, such as offspring provisioning or brood defence. As with brood defence, it is a form of unshared parental investment (Lazarus & Inglis 1986) because it cannot be monopolized by individual members of the brood. However, whereas brood defence involves protecting offspring from an acute threat that can cause complete brood loss, parental resource defences are mounted against more chronic threats that typically cause just partial loss of current breeding success (though not always e.g. Cardoza *et al.* 2006; Welbergen & Davies 2009). Finally, just as with brood provisioning (e.g. Sanz, Kranenbarg & Tinbergen 2000; Hinde 2006 and references therein) and brood defence (e.g. Curio & Onnebrink 1995; Reyer *et al.* 1998; Ruusila & Poysa 1998), parents must cooperate to defend resources for their young from potential rivals but they may well be in conflict over how to share the costs of this parental effort (Chase 1980; Houston, McCleery & Davies 1985; McNamara, Gasson & Houston 1999; McNamara *et al.* 2002; Johnstone & Hinde 2006; Lessells 2006). There has been little previous work, however, to investigate how this burden might be shared (but see Trumbo 2006).

Here, we address this shortcoming by experimentally investigating the antibacterial activities of breeding burying beetles, *Nicrophorus vespilloides* Herbst. *Nicrophorus* beetles require the carcass of a recently dead bird or mouse to breed and parents jointly prepare the carcass by stripping off fur or feathers, rolling it into a ball and burying it (Pukowski 1933; Scott 1998). Beetle larvae hatch in the soil and crawl to the carcass, where they are attended by both parents, who feed them and defend the carcass from competing micro-organisms, maintaining it in a suitable state for larval growth (Rozen *et al.* 2008).

Parent beetles are known to somehow arrest microbial activity on the carcass (Rozen *et al.* 2008). During preparation, adult beetles cover the carcass with oral and anal exudates, and it has been suggested that these are antibacterial in nature (Pukowski 1933; Scott 1998; Rozen *et al.* 2008). Hoback *et al.* (2004) found protein-based antibacterial activity in the saliva of five of the seven *Nicrophorus* species they tested, and in the anal exudates of two species. This activity could be due to inducible antimicrobial peptides (AMPs) or lysozymes, key humoral immune factors that directly kill or inhibit bacterial growth (Boman & Hultmark 1987; Rowley, Brookman & Ratcliffe 1990; Gillespie, Kanost & Tenczek 1997), or phenoloxidases (PO), which have been shown to have antimicrobial activity in insect haemolymph (Boman & Hultmark 1987; Rowley *et al.* 1990; Gillespie *et al.* 1997). However, whilst the PO cascade generates toxic quinines that could inhibit bacterial growth, much of its activity is associated with cell-based immunity, which is unlikely to be useful

in an exudate (Nappi & Vass 1993), suggesting that AMPs or lysozymes are more likely candidates.

In this study, we address three questions: (i) how do parents defend offspring resources from microbial attack? (ii) how is this task shared between them? And (iii) do they respond to the antibacterial activities of their partner?

## Materials and methods

### NICROPHORUS VESPILLOIDES COLONY

The *N. vespilloides* colony was established in May 2005 from wild-caught beetles which had been trapped in Madingley Woods, Cambridge, UK. Wild-caught beetles were added to the colony each subsequent year to maintain genetic diversity. Beetles were maintained in a temperature controlled room at 21 °C. Each male was paired with a non-sibling female and placed in a plastic container (17 × 12 × 6 cm), one-third filled with moist, non-sterile soil and provided with a newly defrosted mouse carcass. The aim was to simulate the microbial conditions the beetles might experience in nature when finding a recently dead carcass. The breeding box was kept in the dark to simulate underground conditions. Around 8–10 days after the parents were mated, their offspring dispersed from the carcass. At this point, larvae were removed from the soil and placed individually in a plastic box (12 × 8 × 2 cm), which was filled with moist soil. Upon reaching adulthood, beetles were fed twice a week on small pieces of minced beef until required for experiments or breeding. Between 50 and 100 pairs successfully produced offspring in each generation. Animals had been reared under standard laboratory conditions for 16 generations at the start of the experiment.

### EXPERIMENT 1: THE EFFECT OF MATING AND CARCASS PRESENCE ON ANAL EXUDATE CONTENT

Upon handling, the majority of beetles produce a brown exudate from their abdomen, which can be easily collected using a glass capillary tube and blown into an eppendorf tube for storage. We tested anal exudates from virgin and mated beetles, either with or without a carcass, for antibacterial activity to ascertain whether it was induced in response to mating or carcass availability.

Beetles were assigned to one of two treatment groups. In each treatment on day 0, beetles were handled, and their exudates collected, before being placed in pairs, one male and one female, into a standard breeding box. In the control treatment, there was no carcass present, in the mouse treatment the breeding box was furnished with a mouse carcass. Exudates were again collected from all beetles in both treatments 2 days after pairing. At this point in a typical breeding event, mating and egg laying have finished but the larvae have yet to arrive at the carcass. Exudates were collected again 8 days after pairing, at which point larvae were almost fully grown and the necessity for direct care by the parents had diminished considerably. Exudates were stored at –20 °C until further analyses were carried out; 50 pairs were established in the mouse treatment, pairs that failed to breed were excluded from the analysis, leaving 43 pairs overall.

### EXPERIMENT 2: THE EFFECT OF EXPERIMENTAL WIDOWING ON ANAL EXUDATE CONTENT

We tested whether beetles could flexibly adjust their antibacterial activity in relation to that of their partner by testing activity in male

and female beetles that were either reared in pairs or that had had their partner experimentally removed. Beetles were assigned to one of three treatment groups: (i) male and female remain with the brood; (ii) male removed 2 days after pairing; or (iii) female removed 2 days after pairing. Again, exudates were collected as the beetles were paired (day 0) and 2 days later. However, for this experiment, the final exudate samples were collected 6 days after pairing, whilst adults were still caring for the offspring. Exudates were stored at  $-20^{\circ}\text{C}$  until further analyses were carried out. Sixty pairs were set up but only beetles that bred successfully were included in the analysis, leaving 44 pairs in total. Of the failures, four were in the paired treatment, six in the widowed female treatment and eight in the widowed male treatment.

#### PHENOLOXIDASE ASSAY

Exudate PO was measured using a modified version of the method described in Cotter, Beveridge & Simmons (2008b). In brief, 1  $\mu\text{L}$  of exudate was added to 100  $\mu\text{L}$  of ice-cold phosphate-buffered saline (PBS, pH 7.4) in a plastic Eppendorf tube and vortexed. PO activity was assayed spectrophotometrically with dopamine as a substrate. This assay involved adding 160  $\mu\text{L}$  of 4 mM dopamine to 40  $\mu\text{L}$  of the buffered exudate and incubating duplicate samples of the mixture on a temperature-controlled Biotek ELX808 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) at 490 nm at  $30^{\circ}\text{C}$ . PO activity was expressed as the change in absorbance over the first 25 min, which is during the linear phase of the reaction.

#### PROTEIN ASSAY

The concentration of PO, or proteins with lysozyme-like activity in the exudate could be driven by natural fluctuations in exudate protein content. Protein levels are expected to rise as the parents feed on the carcass. Therefore, we measured protein levels to assess whether observed variation in antibacterial components could be attributed to these natural fluctuations or whether they were actively regulated. Protein was measured using the BioRad protein assay kit (Bio-Rad Laboratories, Hemel, Hempstead, UK) with BSA as the protein standard. Two replicates of 5  $\mu\text{L}$  of the exudate/PBS mixtures were used to measure the protein in each sample. Absorption was measured on a temperature-controlled Biotek ELX808 microplate reader at 600 nm.

#### LYSOZYME-LIKE ANTIBACTERIAL ACTIVITY

Lytic activity against the bacterium *Micrococcus lysodeikticus* was determined using a lytic zone assay. Agar plates containing 10 mL of 1% agar with 5 mg per mL freeze-dried *M. lysodeikticus* were prepared as described in Kurtz *et al.* (2000). For each plate, 20 holes with a diameter of 2 mm were punched in the agar and 1  $\mu\text{L}$  of exudate was placed in each well, two replicates per sample. The plates were incubated at  $25^{\circ}\text{C}$  for 24 h then photographed using a digital camera. The diameter of the clear zones was calculated using IMAGE J software (<http://rsweb.nih.gov/ij/index.html>). Standard curves were obtained using a serial dilution of hen egg white lysozyme. Concentration of lysozyme-equivalents in mg was then calculated.

#### STATISTICAL ANALYSIS

All analyses were carried out using linear mixed effects REML models in GENSTAT 10 (VSN International, Hemel, Hempstead, UK),

which are more robust with regards to unbalanced designs than ANOVA procedures. As multiple measurements were taken from the same individuals, beetle ID was included as a random effect in both experiments. In Experiment 1, day, sex, carcass treatment and their interactions were included as fixed effects. In Experiment 2, day, sex and partner treatment were included as fixed effects. In addition, the effects of carcass weight, number of larvae in the brood, total brood weight and adult size (pronotum widths of the parents) were included in the models as additional fixed effects but were subsequently removed as they were non-significant. Given the results of previous similar experiments on *Nicrophorus* (e.g. Fetherston, Scott & Traniello 1994; Muller, Eggert & Sakaluk 1998; Smiseth & Moore 2004; Smiseth *et al.* 2005), we predicted there would be sex differences in the response to widowhood. Furthermore, we predicted the exudate constituents would vary with time if their antibacterial activity was actively upregulated following presentation of a carcass. To detect these changes in both Experiments 1 and 2, we predicted a three-way interaction between day, treatment and sex. Where these occurred, the estimated means and standard errors from the full model were examined to ascertain where the differences lay. However, further analyses carried out by splitting the data set where interactions occurred produced extremely similar results.

## Results

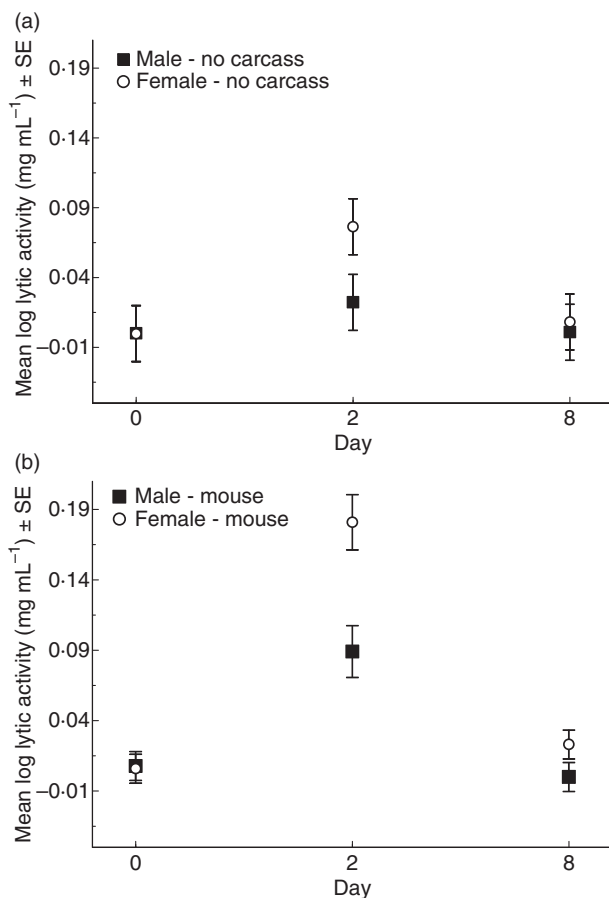
### EXPERIMENT 1: THE EFFECTS OF MATING AND CARCASS PRESENCE ON ANAL EXUDATE CONTENT

#### Lytic activity

There were interactive effects of sex, day and carcass treatment on lytic activity (Table 1). Parameter estimates from the minimal model indicated that exudates sampled from all beetles at pairing (day 0) showed little or no lytic activity (Fig. 1). In fact, only 5 of the 63 beetles for which lytic activity was measured showed any activity at all. Activity then increased by day 2 and decreased again by day 8 (Fig. 1). Parameter

**Table 1.** Experiment 1: results of mixed effects REML model with beetle ID included as a random effect, showing significance of the following fixed effects: sex, day since pairing, carcass treatment (mouse present or absent) and their interactions on lytic activity, PO activity or protein levels in anal exudate. Significant term are highlighted in bold

Fixed term	Lytic activity	PO activity	Protein
Sex	$F_{1,60} = \mathbf{19.23}$ $P < \mathbf{0.001}$	$F_{1,50} = 1.77$ $P = 0.190$	$F_{1,49} = 0.75$ $P = 0.389$
Day	$F_{2,116} = \mathbf{74.3}$ $P < \mathbf{0.001}$	$F_{2,76} = \mathbf{11.07}$ $P < \mathbf{0.001}$	$F_{2,65} = \mathbf{140.49}$ $P < \mathbf{0.001}$
Carcass	$F_{1,71} = \mathbf{12.49}$ $P < \mathbf{0.001}$	$F_{2,52} = \mathbf{4.57}$ $P = \mathbf{0.037}$	$F_{1,52} = \mathbf{83.04}$ $P < \mathbf{0.001}$
Sex $\times$ Day	$F_{2,116} = \mathbf{9.96}$ $P < \mathbf{0.001}$	$F_{2,74} = 0.40$ $P = 0.675$	$F_{2,66} = \mathbf{3.32}$ $P = \mathbf{0.042}$
Sex $\times$ Carcass	$F_{2,73} = 1.39$ $P = 0.243$	$F_{2,51} = 0.04$ $P = 0.841$	$F_{1,51} = 0.80$ $P = 0.374$
Day $\times$ Carcass	$F_{2,122} = \mathbf{7.80}$ $P < \mathbf{0.001}$	$F_{2,77} = \mathbf{5.40}$ $P = \mathbf{0.006}$	$F_{2,69} = \mathbf{55.68}$ $P < \mathbf{0.001}$
Sex $\times$ Day $\times$ Carcass	$F_{2,121} = 0.54$ $P = 0.587$	$F_{2,73} = 0.36$ $P = 0.700$	$F_{2,67} = 1.46$ $P = 0.241$

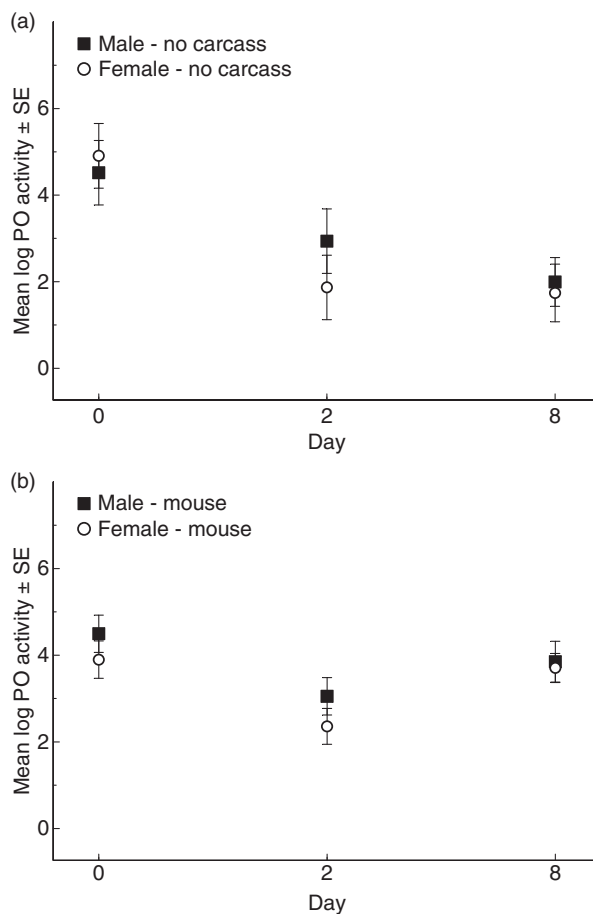


**Fig. 1.** Mean ( $\pm$  SE) lytic activity in anal exudates of male and female beetles on day 0 (i.e. at pairing), day 2 and day 8 (post-breeding) (a) without a carcass and (b) with a carcass. All values displayed are estimates from the minimum adequate model.

estimates indicated that the significant differences between sexes and treatments occurred only on day 2 (Fig. 1). Females provided with a mouse produced significantly higher lytic activity than the other three treatment groups ( $t > 3.415$ , d.f.  $> 22$ ,  $P < 0.003$ ; Fig. 1a,b). Males provided with a mouse and females that did not have a carcass had lytic activity that was significantly  $> 0$  ( $t > 3.82$ , d.f.  $> 7$ ,  $P < 0.005$ ; Fig. 1a,b), whilst males without a carcass did not ( $t_9 = 1.12$ ,  $P = 0.29$ ; Fig. 1a).

#### Phenoloxidase activity

The patterns found for PO activity were quite different to those found for lytic activity. Whereas lytic activity could not be detected in anal exudates at pairing, PO activity levels were high at this point and fell thereafter. There were no significant differences between the sexes in PO activity, but the way in which PO activity changed after pairing depended on whether or not a carcass was present (Table 1). Examination of the model parameter estimates indicated that PO activity levels decreased significantly from day 0 to day 2 in both treatment groups (day 0 vs. day 2:  $t > 2.98$ , d.f.  $> 18$ ,  $P < 0.01$ ; Fig. 2a,b). However by day 8, whilst activity had

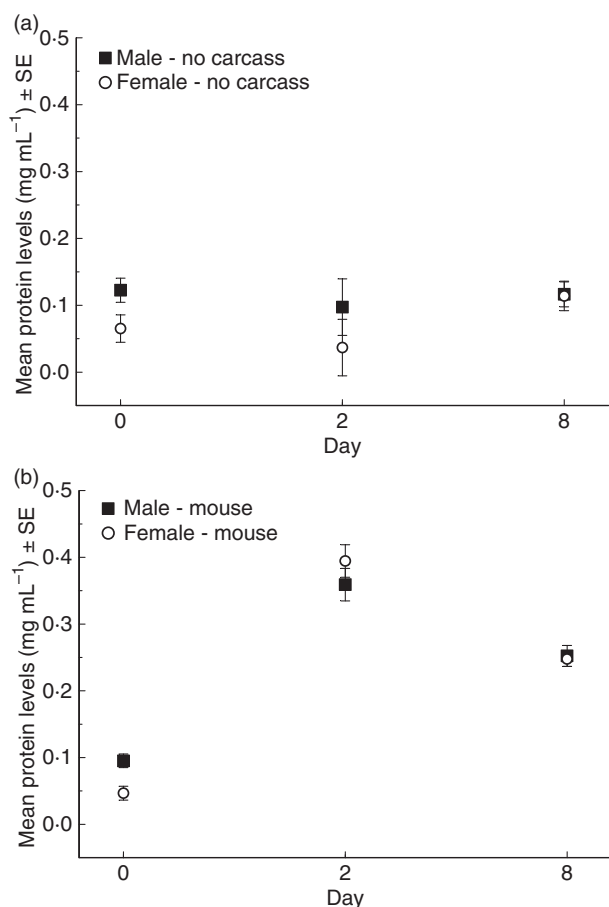


**Fig. 2.** Mean ( $\pm$  SE) phenoloxidase activity in anal exudates of male and female beetles on day 0 (i.e. at pairing), day 2 and day 8 (post-breeding) (a) without a carcass and (b) with a carcass. All values displayed are estimates from the minimum adequate model.

increased again in the beetles that had been provided with a mouse (day 2 vs. day 8:  $t_{53} = 2.63$ ,  $P = 0.01$ ; Fig. 2b), activity remained low in the beetles without a carcass (day 2 vs. day 8:  $t_{18} = -0.73$ ,  $P = 0.48$ ; Fig. 2a).

#### Protein

As for lytic activity, there were interactive effects of sex, day and carcass type on haemolymph protein levels (Table 1). Examination of the model parameter estimates indicated that there were sex differences in exudate protein levels on day 0 with males having higher protein levels than females in both treatment groups ( $t > 2.21$ , d.f.  $> 12$ ,  $P < 0.04$ ). However, the most striking differences were between the treatments after pairing. On day 2, exudate protein levels were much higher in males and females that had been provided with a mouse than in beetles without a carcass ( $t > 5.36$ , d.f.  $> 14$ ,  $P < 0.0001$ ; cf. Fig. 3a,b), and levels remained higher in the mouse treatment by day 8 ( $t > 5.41$ , d.f.  $> 15$ ,  $P < 0.0001$ ; cf. Fig. 3a,b) despite protein levels in that treatment falling relative to day 2 (day 2 vs. day 8:  $t > -3.68$ , d.f.  $> 20$ ,  $P < 0.002$ ; Fig. 3b).



**Fig. 3.** Mean ( $\pm$ SE) protein levels in anal exudates of male and female beetles on day 0 (i.e. at pairing), day 2 and day 8 (post-breeding) (a) without a carcass and (b) with a carcass. All values displayed are estimates from the minimum adequate model.

#### EXPERIMENT 2: THE EFFECTS OF EXPERIMENTAL WIDOWING ON EXUDATE CONTENT

##### Lytic activity

There were interactive effects of day, sex and widowhood treatment on lytic activity (Table 2). As found in Experiment 1, lytic activity levels were negligible at pairing but increased rapidly thereafter in both sexes in all treatment groups (Fig. 4a–c). As would be expected, the model parameter estimates showed that lytic activity levels did not differ by treatment either at pairing ( $t < 0.60$ , d.f.  $> 18$ ,  $P > 0.55$ ), or 2 days later ( $t < 1.46$ , d.f.  $> 15$ ,  $P > 0.16$ ). However, it can be seen that by day 6 there were differences between the treatment groups and the sexes (Fig. 4a–c).

Females who had been taken off the carcass had lower lytic activity levels than paired females ( $t_{23} = 4.33$ ,  $P < 0.001$ ; cf. Fig. 4a,c), whereas widowed females showed intermediate lytic activity (widowed vs. paired:  $t_{25} = -2.28$ ,  $P = 0.031$ ; widowed vs. removed:  $t_{20} = 2.14$ ,  $P = 0.045$ ; cf. Fig. 4a–c). The response of males was different to that of females; 6 days after pairing, widowed males had higher activity levels than either paired males ( $t_{21} = 2.19$ ,  $P = 0.040$ ; cf. Fig. 4a,c) or

**Table 2.** Experiment 2: results of mixed effects REML models with beetle ID included as a random effect, showing significance of the following fixed effects: sex, day, parent widowhood treatment and their interactions on either lytic activity, PO activity or protein levels in anal exudate. Significant terms are highlighted in bold

Fixed term	Lytic activity	PO activity	Protein
Sex	$F_{1,80} = 7.05$ $P = 0.010$	$F_{1,83} = 0.25$ $P = 0.615$	$F_{1,76} = 3.56$ $P = 0.063$
Day	$F_{2,147} = 101.21$ $P < 0.001$	$F_{2,116} = 9.09$ $P < 0.001$	$F_{2,135} = 37.31$ $P < 0.001$
Treatment	$F_{2,79} = 2.52$ $P = 0.087$	$F_{2,76} = 0.12$ $P = 0.888$	$F_{2,69} = 0.38$ $P = 0.686$
Sex $\times$ Day	$F_{2,153} = 3.66$ $P = 0.028$	$F_{2,124} = 5.37$ $P = 0.006$	$F_{2,143} = 3.76$ $P = 0.026$
Sex $\times$ Treatment	$F_{2,77} = 3.96$ $P = 0.023$	$F_{2,75} = 0.98$ $P = 0.380$	$F_{2,58} = 0.10$ $P = 0.903$
Day $\times$ Treatment	$F_{4,152} = 3.78$ $P = 0.006$	$F_{4,111} = 1.94$ $P = 0.108$	$F_{4,132} = 0.43$ $P = 0.789$
Sex $\times$ Day $\times$ Treatment	$F_{4,142} = 2.39$ $P = 0.054$	$F_{4,109} = 0.37$ $P = 0.832$	$F_{4,128} = 0.21$ $P = 0.933$

males that had been removed from the carcass ( $t_{22} = 2.48$ ,  $P = 0.021$ ; cf. Fig. 4b,c). The latter two groups did not differ significantly from each other ( $t_{23} = 0.29$ ,  $P = 0.77$ ; cf. Fig. 4a,b).

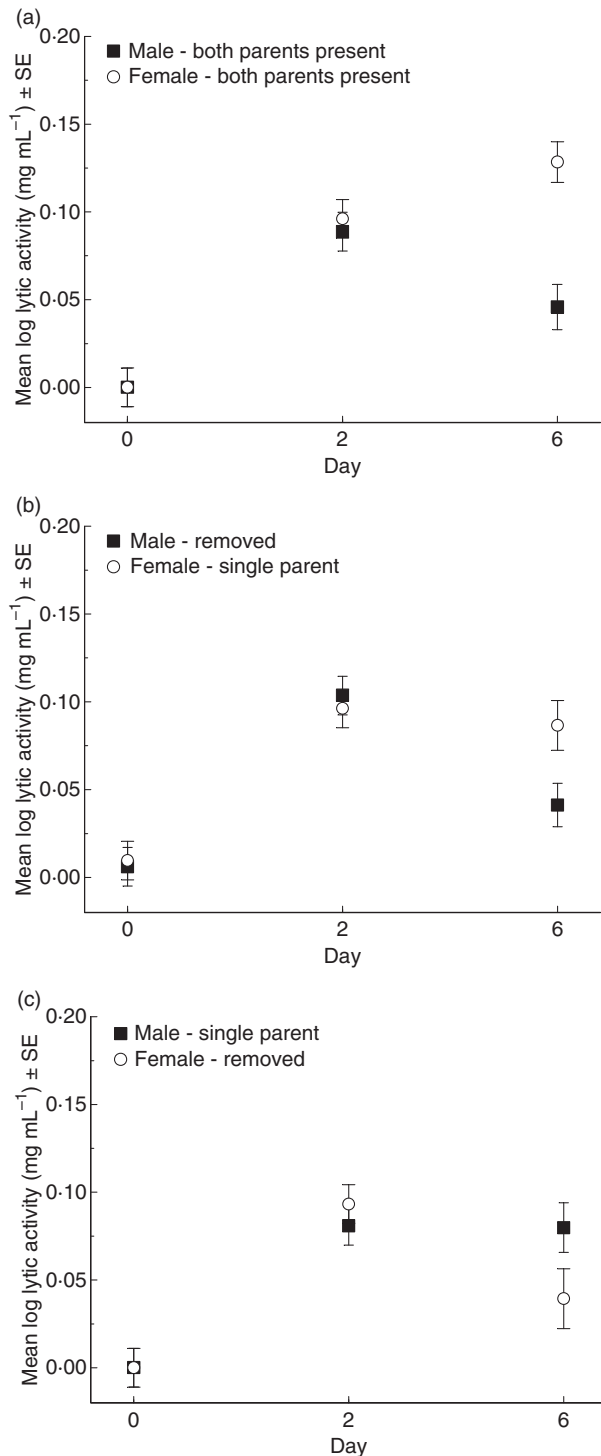
When pairs remained together, females had significantly higher lytic activity than males ( $t_{25} = 5.31$ ,  $P < 0.0001$ ; Fig. 4a), and this was also true when comparing widowed females with the male that had been removed ( $t_{25} = 2.92$ ,  $P = 0.007$ ; Fig. 4b). Conversely, widowed males had higher lytic activity than their partners that had been removed from the carcass ( $t_{17} = 2.60$ ,  $P = 0.019$ ; Fig. 4b). However, there was no significant difference between the lytic activity of widowed males and widowed females ( $t_{21} = 0.44$ ,  $P = 0.67$ ; cf. Fig. 4b,c).

##### Phenoloxidase activity

The sexes showed differing levels of PO activity in the days following pairing, but this did not differ in response to the widowhood treatment (Table 2). The parameter estimates from the model show that PO activity was higher in females than males at pairing ( $t_{49} = 2.99$ ,  $P = 0.004$ ; Fig. 5a), but there was no difference between the sexes 2 or 6 days after pairing ( $t < 1.02$ , d.f.  $> 56$ ,  $P > 0.32$ ; Fig. 5a). This is because PO activity levels in females fell after pairing (day 0 vs. day 2:  $t_{58} = 3.41$ ,  $P = 0.001$ ; Fig. 5a), and remained low (day 2 vs. day 6:  $t_{52} = 1.35$ ,  $P = 0.18$ ; Fig. 5a), whereas PO activity levels in males did not differ significantly across days ( $t < 1.12$ , d.f.  $> 46$ ,  $P > 0.27$ ; Fig. 5a).

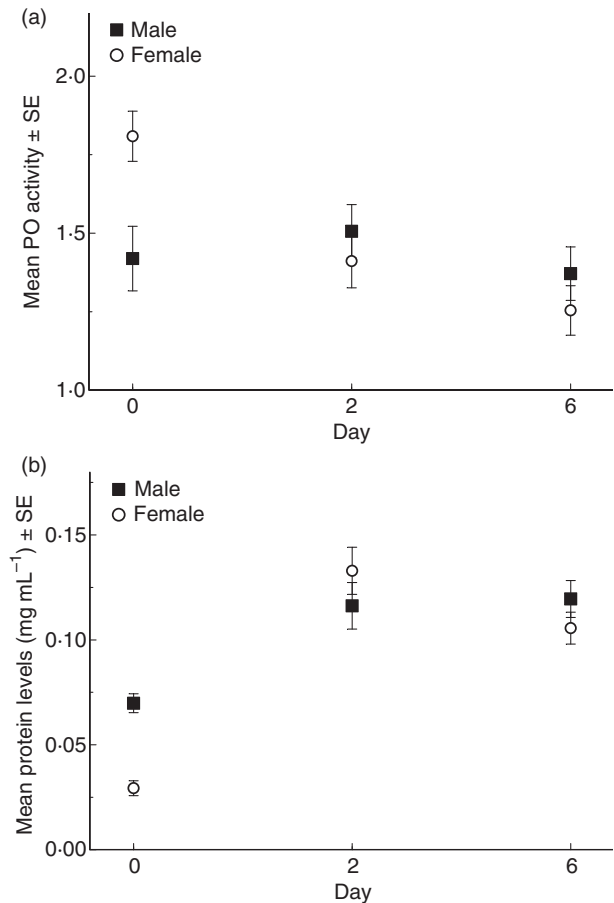
##### Protein

Just as for PO activity, the sexes showed differing levels of protein in the days following pairing, but did not differ in their response to the widowhood treatment (Table 2). The parameter estimates from the model showed that males had



**Fig. 4.** Mean ( $\pm$  SE) lytic activity in anal exudates of male and female beetles at pairing, with a carcass present (day 0) and 2 and 6 days later where (a) both parents were present to rear the brood; (b) males were removed 2 days after pairing; (c) females were removed 2 days after pairing. All values displayed are estimates from the minimum adequate model.

significantly higher exudate protein levels than females at pairing, just as in Experiment 1 ( $t_{49} = 7.25$ ,  $P < 0.0001$ ; Fig. 5b), but 2 and 6 days later, there were no sex differences in protein levels ( $t > 1.09$ , d.f.  $> 49$ ,  $P > 0.25$ ; Fig. 5b).



**Fig. 5.** Mean ( $\pm$  SE) (a) phenoloxidase activity and (b) protein levels in anal exudates of males and females at pairing with a carcass present (day 0) and 2 and 6 days later. Data for the widowing treatments are combined as their effect was non-significant. All values displayed are estimates from the minimum adequate model.

Female protein levels changed in the days after pairing: levels on day 2 and day 6 were higher than on day 0 (day 2 vs. day 0:  $t_{57} = 8.89$ ,  $P < 0.0001$ ; day 6 vs. day 0:  $t_{63} = 8.61$ ,  $P < 0.0001$ ; Fig. 5b). The pattern was the same in males (day 2 vs. day 0:  $t_{46} = 3.93$ ,  $P < 0.001$ ; day 6 vs. day 0:  $t_{46} = 5.08$ ,  $P < 0.0001$ ; Fig. 5b). In both sexes, levels on days 2 and 6 were not significantly different from each other ( $t < 1.99$ , d.f.  $> 54$ ,  $P > 0.05$ ; Fig. 5b).

## Discussion

Our experiments show that parents coat offspring resources with antibacterial anal exudates to defend them from microbial competitors, thus substantiating a long-standing, yet hitherto untested, claim (e.g. Pukowski 1933; Bartlett & Ashworth 1988; Scott 1998); but see (Hoback *et al.* 2004). Both sexes contributed to this endeavour, although a female's exudates typically showed higher levels of antibacterial activity than those of her mate (Figs 1b and 4a). Furthermore, parents flexibly adjusted their antibacterial activity in response to that of their partner. Males increased the antibacterial concentration of their exudates after females were

removed, while widowed females reduced their antibacterial activity (Fig. 4).

#### ANAL EXUDATES AS AN EXTERNAL COMPONENT OF THE INSECT IMMUNE SYSTEM

We found that the presence of a carcass was necessary to induce antibacterial activity in the anal exudates. This might explain why Hoback *et al.* (2004) found relatively little antibacterial activity in the exudates of several field-caught *Nicrophorine* beetles, and related species, of unknown breeding status. Perhaps, these species would have upregulated their antibacterial activity had they been presented with a carcass, as we did here.

The upregulation of lysozyme-like antibacterial activity in the anal exudates following presentation of a carcass (Figs 1b and 4a–c) is similar to the upregulation of constitutively present lysozymes in the haemolymph following infection (e.g. Anderson & Cook 1979; Boman & Hultmark 1987; Korner & Schmid-Hempel 2004; Haine *et al.* 2008). This, and other similarities with the invertebrate immune response outlined below, means that the anal exudates can be thought of as an external component of the beetle's immune system. Just as in the insect immune system, the anal exudates appear to have several elements that may act together in carcass defence. Anal exudates had PO as well as lytic activity. However, unlike lytic activity, PO activity was downregulated in response to the presence of a carcass. It is possible that this was not an adaptive response, but a constraint due to a trade-off between PO and lytic expression, similar to the trade-offs known to occur between these components of the immune system in several different insect species (Moret & Schmid-Hempel 2001; Rantala & Kortet 2003; Cotter, Kruuk & Wilson 2004; Freitak *et al.* 2007; Cotter *et al.* 2008a; Povey *et al.* 2009). In general, neither lytic nor PO activity could be explained by variation in overall protein levels (cf. Figs 4 and 5), which rules out the possibility that beetles were simply increasing the concentration of the exudates following presentation of the carcass. Instead, it suggests that beetles actively regulate the composition of their anal exudates.

What mechanisms might induce antibacterial activity in the anal exudates? Juvenile hormone (JH) levels increase rapidly in both sexes of *Nicrophorus orbicollis* upon finding a carcass (Scott & Panaitof 2004) and, in other insect species, high levels of JH downregulate PO activity (Rolff & Siva-Jothy 2002) and might even regulate the trade-off between PO and lysozyme activity found in the haemolymph (Wilson & Cotter 2009). It is possible therefore that JH is responsible for the upregulation of lytic activity in anal exudates following the discovery of a carcass. An additional potential trigger could be the oral detection of micro-organisms, because this is known to upregulate antibacterial activity and downregulate PO activity in the haemolymph of the Lepidopteran cabbage looper, *Trichoplusia ni* (Freitak *et al.* 2007).

#### SEX DIFFERENCES IN CONTRIBUTIONS TO SOCIAL IMMUNITY

The external immune function shown by the anal exudates represents a novel form of social immunity (Cremer, Armitage & Schmid-Hempel 2007), because it involves the collective defence of a breeding resource against attack by bacteria. Both sexes gain by mounting a social immune defence (Rozen *et al.* 2008), but it is interesting to ask how this task is divided between them.

Sex differences in the internal invertebrate immune response are well known from previous work (e.g. females greater than males: Kurtz *et al.* 2000; Adamo, Jensen & Younger 2001; Cotter *et al.* 2008a; males greater than females: McKean & Nunney 2005; Rantala, Roff & Rantala 2007). We found similar sex differences in the contributions made to social immunity. As with some other aspects of parental care (Scott 1989, 1990; Fetherston, Scott & Tranello 1990; Fetherston *et al.* 1994; Muller *et al.* 1998; Jenkins, Morris & Blackman 2000; Smiseth & Moore 2004; Smiseth *et al.* 2005), females showed consistently higher levels of antibacterial activity than males (Figs 1 and 4a). Perhaps, females take primary responsibility for protecting the carcass from bacteria, whilst males put more effort into cleaning the carcass, maintaining the crypt or defending the carcass and the offspring from predators and congeneric usurpers (Scott 1990). Such sex-specific roles in the defence of offspring resources have been described in a number of other species that show biparental care (Palomino *et al.* 1998; Gill & Sealy 2004; Rasa & Heg 2004).

We tested whether parents could flexibly adjust their defence of offspring resources when experimentally widowed. There was no effect of widowhood on either PO activity or overall protein levels in either sex, each following similar patterns to those found in the first experiment (Fig. 5). The results for lytic activity were quite different, though. Six days after pairing, control paired males reduced their levels of lytic activity so much that they matched those of individuals that had been removed from a carcass (cf. Fig. 4a,b). Meanwhile their partners exhibited very high levels of lytic activity (Fig. 4a). However, when males were experimentally widowed 2 days after pairing, their levels of lytic activity were still high 4 days later (Fig. 4c). Nevertheless, they only partially compensated for the female's absence, failing to produce the high levels of lytic activity seen in the exudates of paired females (cf. Fig. 4a,c). From a mechanistic perspective, it is interesting that these changes in lytic activity match changes in JH levels seen when *N. orbicollis* males are experimentally widowed: low JH levels are seen in paired males but JH titres increase when males are widowed after larvae hatch (Panaitof, Scott & Borst 2004).

When we experimentally widowed females, we found a different response, with females reducing antibacterial activity in their exudates, compared with paired females (cf. Fig. 4a,b). As a result, widowed parents of each sex produced exudates of comparable lytic activity. Previous work on the

post-hatching parental care of burying beetles has revealed broadly similar responses to widowhood that also differ between the sexes. Widowed males increase their provision of care, this time fully compensating for the female's absence (Fetherston *et al.* 1994), while widowed females do not alter their caring behaviour in response to male removal (Smiseth *et al.* 2005). Sex differences in the way that parents respond to their partner's parental effort have also been documented in birds (e.g. Sanz *et al.* 2000).

How can we account for the contrasting responses of the male and female beetles in our experiments to widowhood? We consider two possibilities that are not mutually exclusive. The first is that males are functionally semelparous and females iteroparous, resulting in males placing more value in the current brood than females. Males reproducing in nature have greater variance in reproductive success per breeding attempt, and are less likely than females to gain reproductive success as subordinates on a carcass (Muller *et al.* 2007), which lends some support to this idea. Standing against this hypothesis, however, are laboratory studies on *N. orbicollis* (Scott 1998) and *N. vespilloides* (Ward 2007), which show that female reproductive success decreases with later breeding attempts, while male reproductive success does not.

A second possibility is that a high level of task specialization within each sex accounts for our results (Fetherston *et al.* 1990; Muller *et al.* 1998; Trumbo 2006). Though males and females share the same repertoire of parental behaviours (Smiseth & Moore 2004), it has been shown that during brooding females spend more time caring for larvae and processing carrion and males spend more time guarding and maintaining carrion (Smiseth & Moore 2004). However, parents may take on different roles in carcass maintenance, with females primarily responsible for producing antibacterial exudates, and males spending more time cleaning the carcass and maintaining the crypt. Perhaps, in the absence of the female, the male partially compensates by upregulating his activity to the minimum required for carcass maintenance, but cannot fully compensate due to the requirement to fulfil other duties. Similarly, in the absence of the male, the female may have to take on a larger share of these other tasks, the extra cost of which reduces the investment she can put into lytic activity. This explanation for our results this seems most compatible with what we currently know about *Nicrophorus* breeding biology.

To conclude, we have shown that anal exudates are an external component of the beetle's immune system, and serve a social immune function by defending the carcass against bacteria. Parents flexibly share the task of protecting the breeding resource in this way, just as they have previously been shown to share other aspects of parental care, such as offspring provisioning and brood defence. The challenge for future work is investigate how parents balance contributions to social immunity with their own immune defence, and to identify the hormonal mechanisms governing any such trade-off.

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## Author contributions

SC designed and executed the experiments and analysed the data; SC and RMK co-wrote the paper.

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