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LETTER

Fitness costs associated with mounting a social immune response

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Abstract

Social immune systems comprise immune defences mounted by individuals for the benefit of others (*sensu* Cotter & Kilner 2010a). Just as with other forms of immunity, mounting a social immune response is expected to be costly but so far these fitness costs are unknown. We measured the costs of social immunity in a sub-social burying beetle, a species in which two or more adults defend a carrion breeding resource for their young by smearing the flesh with antibacterial anal exudates. Our experiments on widowed females reveal that a bacterial challenge to the breeding resource upregulates the antibacterial activity of a female's exudates, and this subsequently reduces her lifetime reproductive success. We suggest that the costliness of social immunity is a source of evolutionary conflict between breeding adults on a carcass, and that the phoretic communities that the beetles transport between carrion may assist the beetle by offsetting these costs.

Keywords

Antimicrobial, cooperation, ecological immunity, mite, phoresy, sexual conflict, social insect.

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INTRODUCTION

Many organisms defend their fitness against attack from parasites and pathogens by mounting an immune response. In addition to the well-known personal immune system, some immune defences are deployed for the benefit of others, besides the challenged individual. For example, some fish apply antimicrobial secretions onto their eggs (Knouft *et al.* 2003; Giacomello *et al.* 2006) or to the nest surface (Little *et al.* 2008) for the benefit of their developing young. Metapleural gland secretions in leafcutter ants are deployed against fungi and bacteria that compete with their symbiotic fungus (Nascimento *et al.* 1996). Similarly, termites and bark beetles coat the inside of their chambers respectively with antifungal faecal pellets and oral secretions (Rosengaus *et al.* 1998; Cardoza *et al.* 2006). In the latter examples, activation of the immune system brings benefits to neighbouring conspecifics and even heterospecific members of the community (Fernandez-Marin *et al.* 2009), as well as the immune-challenged individual. Whenever individuals mount an immune response for the benefit of others as well as themselves, these are examples of social immunity. (Note that our definition of the term 'social immunity' is slightly broader than current one and readers may wish to refer to Cotter & Kilner (2010a), where we provide a full justification for our terminology.)

According to life history theory, maintaining any sort of immune response will come at a cost (Sheldon & Verhulst 1996). Identifying the nature and magnitude of these costs, and the trade-offs they create, is central to understanding the evolution of immune systems (Sheldon & Verhulst 1996; Schulenburg *et al.* 2009). Consistent with this approach, there is experimental evidence of costs associated with mounting a personal immune response (Ilmonen *et al.* 2000; Moret & Schmid-Hempel 2000; Siva-Jothy & Thompson 2002; Bonneaud *et al.* 2003; Jacot *et al.* 2004). Just as with the personal immune response, mounting a social immune response is also likely to be costly because it diverts resources away from other functions (Lochmiller & Deerenberg 2000). Indirect evidence that this is the case comes from the observation that social immune responses are often upregulated only in response to a specific challenge (e.g. Fernandez-Marin *et al.* 2009; Cotter & Kilner 2010b). Furthermore, where aspects of social immunity are constitutively expressed (examples in Cremer *et al.* 2007; Fernandez-Marin *et al.* 2009), they are most typically shown by the non-reproductive members of complex insect societies (Cremer *et al.* 2007). However, whether there are any fitness

costs directly associated with mounting a social immune response remains unclear, partly because these immune responses are typically studied in social insects, where sterile workers are responsible for social immunity (Cremer *et al.* 2007). Nevertheless identifying any fitness costs associated with responding to a social immune challenge is important, not only for understanding related life history trade-offs, but also because it potentially classifies social immunity as a form of cooperation (Hamilton 1964), with the challenged individual providing a benefit to another individual at some cost to itself.

Here, we measure the fitness costs of mounting a social immune response in the sub-social burying beetle *Nicrophorus vespilloides* Herbst. Burying beetles exhibit elaborate biparental care, with duties including the preparation and maintenance of a carcass to sustain their developing young. They also defend the carcass and larvae from predators and competitors, and feed their developing young with partially digested flesh from the corpse (Pukowski 1933). Carcass preparation involves stripping a small vertebrate corpse of fur or feathers, rolling it into a ball, burying it in an underground chamber and covering it in anal and oral exudates (Eggert & Müller 1997; Scott 1998). Although these exudates have long been assumed to serve an antimicrobial function, direct evidence to show that this is the case has only recently been obtained (Cotter & Kilner 2010b).

This recent work shows that the anal exudates that parents smear on the carcass have features in common with the immune function of insect haemolymph because they exhibit lysozyme-like and phenoloxidase (PO) activity, which appear to trade-off against each other (Cotter & Kilner 2010b), an effect that has been shown in the internal immune system in many different insect species (Moret & Schmid-Hempel 2001; Rantala & Kortet 2003; Freitak *et al.* 2007; Cotter *et al.* 2008a; Povey *et al.* 2009). Whilst exudate lysozyme-like activity is upregulated and facultatively adjusted upon discovery of a carcass, PO activity is downregulated, suggesting that PO does not function as a preservative on the carcass (Cotter & Kilner 2010b). The anal exudates protect resources on the carcass from microbial competitors whose presence can dramatically reduce larval fitness (Rozen *et al.* 2008). The anal exudates are therefore part of a social immune defence (Cotter & Kilner 2010a), functioning in exactly the same way as the metapleural gland secretions of worker ants (see section 3 in table 1 of Cremer *et al.* (2007)) to promote 'nest' hygiene for the benefit of larvae. In the burying beetle, additional beneficiaries of this collective immune defence include the beetle's mate and any unrelated adults, whose larvae are raised on the same carcass [brood parasitism or joint breeding occur in more than half of natural breeding attempts (Müller *et al.* 2007)]. For all these reasons, the burying beetle's anal exudates constitute part of a social immune system.

Both parents take part in carcass preparation, but antibacterial exudates are primarily produced by the female in *N. vespilloides*, although the male can partially compensate if females are removed (Cotter & Kilner 2010b). Our previous work suggests that maintaining high levels of antibacterial activity in the anal exudates is likely to be costly. Antibacterial activity is not constitutively expressed but induced by the presence of a carcass. Once a carcass is discovered, lysozyme-like antibacterial levels increase rapidly over 2 days and remain high until the larvae disperse from the carcass. In addition, if either parent is removed from the carcass before breeding is completed the antibacterial levels in their anal exudates fall rapidly (Cotter & Kilner 2010b).

Burying beetles offer the ideal opportunity to quantify fitness costs associated with social immune responses because, unlike in many social insect species, reproductive adults contribute to social immunity. Here, we reveal fitness costs directly associated with mounting this social immune response, that are independent of any other lifetime reproductive costs associated with parental care (Ward *et al.* 2009). Our experiments involve exposing females to carcasses that have been bacterially challenged and then monitoring their subsequent lifetime breeding success and survival.

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 beetles were collected from Byron's Pool local nature reserve, Cambridge, UK each subsequent year during August and added to the colony to maintain genetic diversity. Beetles were reared in a temperature-controlled room at 21 °C with a 16 : 8 light : dark cycle. Unrelated pairs were 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 (10.82 ± 0.2 g). The breeding box was kept in the dark to simulate underground conditions. Offspring disperse from the carcass *c.* 8 days after the parents have been paired. At this point, larvae were removed from the soil and placed individually in plastic boxes (12 × 8 × 2 cm) 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 150 pairs successfully produced offspring each generation. Animals had been reared under standard laboratory conditions for 20 generations at the start of the experiment. For all experiments, beetles were *c.* 2 weeks old at first mating (mean ± SE age in days = 15.13 ± 0.37).

Experiment 1: Forced upregulation of exudate antibacterial activity

Before attempting to uncover any fitness costs associated with induced antibacterial activity in the anal exudates, we first had to develop a technique to force females to upregulate their antibacterial activity to a higher level than would normally be required on a fresh carcass. To do this, we decided to simulate experimentally the natural situation of a female finding a carcass that was in an advanced state of decay. A potential problem here is that a decaying carcass offers a poorer quality resource for larval growth (Rozen *et al.* 2008), and so any subsequent effects on lifetime reproductive success could be due to the quality of the carcass rather than altered behaviour by the parents. To overcome this difficulty, we dipped fresh carcasses in an overnight culture of *Micrococcus lysodeikticus* in nutrient broth that was either live (live bacteria) or that had been autoclaved (dead bacteria). *M. lysodeikticus* is a soil bacterium that does not degrade the quality of the carcass but its presence on the carcass should indicate to the female that the corpse is bacterially compromised. Although the bacteria do not compromise survival (see Results) it is possible that they have sublethal effects on reproduction. Therefore, we used the heat-killed bacterial treatment as a way of upregulating antibacterial exudates whilst controlling for any potential negative effects on the beetle of an actively replicating bacterium. As a positive control, carcasses were dipped in sterile nutrient broth whilst the negative control carcasses were left unmanipulated. Virgin females were then each placed on a carcass in a breeding box with a virgin male and left to prepare the carcass for breeding. After 2 days, exudates were sampled from all females and stored at −20 °C until further analyses were carried out.

Upon handling, the majority of our laboratory 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. Lytic activity in the exudates against the bacterium *M. lysodeikticus* was determined using a lytic zone assay. Agar plates were prepared containing 10 mL of 1% agar with 5 mg per mL freeze-dried *M. lysodeikticus*. For each plate, 20 holes with a diameter of 2 mm were punched in the agar and 1 μL of exudate was placed in each well, two replicates per sample. The plates were incubated at 25 °C for 24 h then photographed using a digital camera. The diameter of the clear zones was calculated using Image J software. Standard curves were obtained using a serial dilution of hen egg white lysozyme and the concentration of lysozyme in mg per mL was then calculated.

Additionally, PO activity was measured in the exudates using a modified version of the method described in Cotter *et al.* (2008b). In brief, 1 μL of exudate was added to 100 μL of ice-cold phosphate buffered saline (pH 7.4) in a plastic eppendorf tube and vortexed. This assay involved adding 100 μL of 4 mM dopamine to 45 μL 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 °C. PO activity was expressed as the change in absorbance over the first 15 min, which is during the linear phase of the reaction.

Experiment 2: Survival costs of antibacterial upregulation after a single breeding bout

Having developed a technique for upregulating antibacterial activity in the anal exudates, we next investigated whether antibacterial upregulation was associated with any subsequent survival costs, in the absence of further reproductive attempts. Virgin females were assigned to one of three treatment groups: control, broth-dipped or bacteria-dipped carcasses as described above. For each treatment, 42 pairs were established. Of these, there were 26 successful breeding pairs in the control treatment, 27 successful pairs in the broth-dipped carcass treatment and 23 successful pairs for the bacteria-dipped carcass treatment. Each female was paired with an unrelated virgin male, and both were placed on a carcass in their own breeding box. All males were removed after 24 h. Females were then left to prepare the carcass and raise offspring. At dispersal, each larva was weighed and the number of dispersing larvae for each female noted. Females were then placed back into individual containers and fed twice weekly until death.

Females may be able to recoup the costs of antibacterial upregulation if food is provided *ad libitum* throughout the remainder of their lives. If this is the case then any survival costs associated with mounting a social immune response may only be apparent during starvation. To test this possibility, a further 60 pairs of virgin beetles were established, 30 with broth-dipped and 30 with bacteria-dipped mice. Of these, 21 pairs in the bacteria treatment and 22 pairs in the broth treatment bred successfully. Females were allowed to breed in these two treatments exactly as described above but after breeding, females were kept without food until they died.

Experiment 3: Survival and fecundity costs of antibacterial upregulation with multiple breeding bouts

This experiment was designed to test first, whether there were fecundity costs associated with antibacterial upregulation and second, whether survival costs of upregulation were apparent if females were given the opportunity to reproduce throughout their life. This experiment was carried out in two replicates. In the first replicate, virgin

females were assigned to one of two treatments: breeding with either a sterile broth-dipped carcass or a carcass that had been dipped in live bacteria for their first two broods. In the second replicate, a further dead bacteria treatment was included to rule out the possibility that any costs were due to pathogenic effects of the bacteria. For their subsequent breeding attempts, females in each treatment were allowed to breed on untreated carcasses, with 3 days to rest between breeding bouts, and they were induced to breed repeatedly until they died. For each breeding attempt, females were paired with a new young, virgin male and larvae were collected at dispersal, weighed and counted. In total, 105 pairs were set up but 7 of the pairs failed to reproduce in any attempt (2 sterile, 3 dead and 2 live bacteria) and so were removed from the analyses, leaving 98 pairs in total.

Statistical analyses

The longevity data were analysed using age-specific, parametric survival models in S-Plus 7 (Tibco, Basingstoke, UK) using a Weibull distribution, which assumes that the risk of death increases with age. All other data were analysed using linear mixed effects Restricted Estimate Maximum Likelihood (REML) models in Genstat 10 (VSN International, Hemel Hempstead, UK) with the family from which the focal beetle originated included as a random effect. In the analysis of repeated breeding, we first checked that there were no significant differences between the replicates for the live bacteria and broth-dipped treatments ($F_{1,408} < 2.28$, $P > 0.13$); data for the two replicates were then pooled for the final analysis and the identity of the pair was also included as a random effect. In all models, the weight of the carcass that beetles bred on was included as a covariate, because carcass weight influences the size and number of offspring reared (see Experiment 2) and because carcass size might be a confounding influence on the upregulation of antibacterial activity in the anal exudates. Estimation of the goodness-of-fit of a mixed effects REML model is not straightforward as there is no equivalent statistic to the r^2 available. Therefore, to estimate the goodness-of-fit of our models, fitted values from each of the models were regressed against the original data and an r^2 of this regression is reported. Means ± standard errors are reported throughout.

RESULTS

Experiment 1: Forced upregulation of exudate antibacterial activity

Presenting female beetles with bacterially challenged carcasses successfully forced them to upregulate their lysozyme activity to higher levels than would typically be seen when breeding on a fresh carcass. There was a significant effect of carcass treatment on lytic activity in the females' anal exudates ($F_{3,131} = 14.05$, $P < 0.001$; Fig. 1). Dipping the carcass significantly upregulated lysozyme activity over undipped controls ($t_{82} > 3.9$, $P < 0.001$). Amongst the dipped carcasses, females breeding on a bacteria-dipped carcass had significantly greater levels of lytic activity in their anal exudates than females breeding on a sterile broth-dipped carcass ($t_{125} > 2.546$, $P < 0.012$; Fig. 1). However, exudate activity did not differ significantly between females breeding on live or dead bacteria-dipped carcasses ($t_{112} = 1.004$, $P = 0.32$; Fig. 1) and PO activity did not differ between any of the treatment groups ($F_{2,79} = 1.25$, $P = 0.29$).

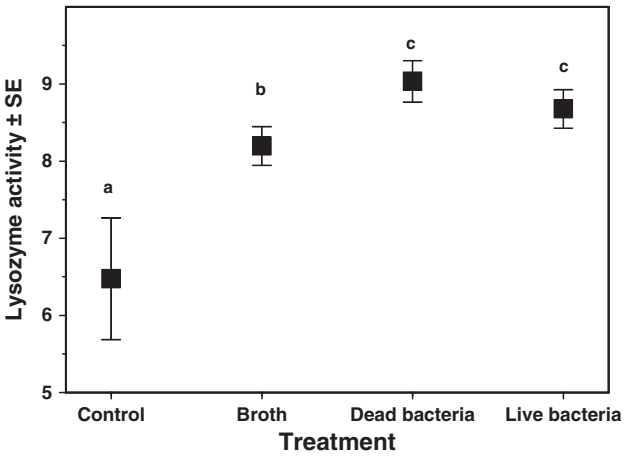

Figure 1 Experiment 1: Mean (± SE) lytic activity measured in exudates collected from females that had been presented with a carcass that had either been untreated (control), dipped in sterile nutrient broth (broth) or dipped in a solution of heat-killed (dead bacteria) or live *Micrococcus lysodeikticus* cells in nutrient broth (live bacteria).

Experiment 2: Survival costs of antibacterial upregulation after a single breeding bout

When females bred just once in their lives, we could detect no survival costs associated with antibacterial upregulation. Females that bred on a bacteria-dipped carcass survived at a similar rate as those that had bred on either sterile broth-dipped or control carcasses (minimal model containing treatment only: $\chi^2_2 = 3.32$, $P = 0.19$). Survival rates were also unrelated to the weight of the mouse carcass used for breeding ($Z_{83} = -1.029$, $P = 0.30$), female age at mating ($Z_{83} = 0.187$, $P = 0.85$), the number of offspring produced ($Z_{83} = -1.033$, $P = 0.30$) or the brood's total mass ($Z_{83} = -0.676$, $P = 0.50$). One possible explanation for these non-significant results is that females recoup any survival costs

associated with antibacterial upregulation by feeding after breeding. However, when we analysed the survival of females that were starved after breeding, we again found no significant effect of carcass treatment on female survival (minimal adequate model containing treatment only: $\chi^2_2 = 0.27$, $P = 0.99$).

We wondered whether females might have shunted any costs associated with upregulated antibacterial activity onto the current brood, by skimping on post-hatching care. However, we could detect no effect of carcass treatment on either the number of larvae produced ($F_{2,129} = 1.89$, $P = 0.156$), the total brood mass ($F_{2,120} = 0.53$, $P = 0.587$), or on the average larval mass at dispersal ($F_{2,120} = 1.16$, $P = 0.316$). Nevertheless, there were positive effects of carcass weight on each of the offspring traits we measured (number of larvae: $F_{1,123} = 4.07$, $P = 0.046$; total brood mass: $F_{1,119} = 32.18$, $P < 0.001$; average larval mass at dispersal: $F_{1,74} = 30.66$, $P < 0.001$).

Experiment 3: Survival and fecundity costs of antibacterial upregulation with multiple breeding bouts

Forcing females to breed repeatedly throughout their lives uncovered a survival cost associated with mounting a social immune response. Females that had bred on bacteria-dipped carcasses in the first two breeding attempts subsequently died at a faster rate than females breeding on control carcasses (live bacteria vs. sterile broth: $Z_{94} = 3.89$, $P < 0.001$, dead bacteria vs. sterile broth: $Z_{94} = 4.92$, $P < 0.001$; Fig. 2). Females breeding on carcasses dipped in dead bacteria also died more quickly than females breeding on carcasses dipped in live bacteria ($Z_{62} = 4.55$, $P < 0.001$; Fig. 2). In addition, females that bred on heavier carcasses on average throughout their lives also died at a faster rate, independent of the carcass bacterial treatment ($Z_{94} = 3.99$, $P < 0.001$). Survival was influenced by the number of offspring that females produced during the first two broods ($Z_{94} = 2.37$, $P = 0.017$), but not by the mass of offspring produced from these broods ($Z_{94} = 0.987$, $P = 0.32$).

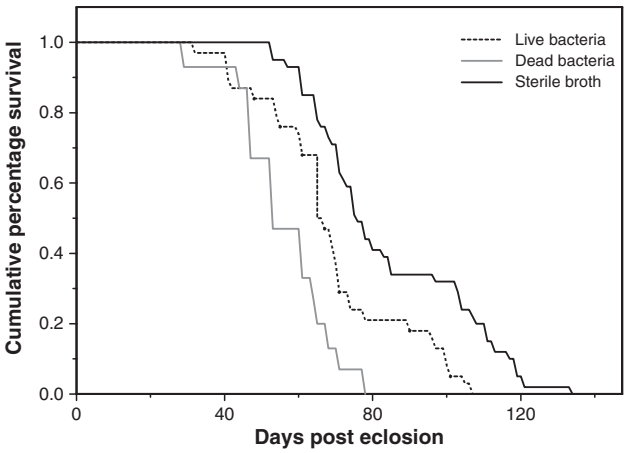

Figure 2 Experiment 3: Cumulative percentage survival for females that had bred twice on a carcass that had either been dipped in nutrient broth (sterile broth) or dipped in a solution of heat-killed (dead bacteria) or live *Micrococcus lysodeikticus* cells in nutrient broth (live bacteria). Females were then repeatedly bred on untreated carcasses until death.

There was a marginally non-significant trend for females that had bred on live bacteria-dipped carcasses to produce fewer successful broods during their lifetime [mean (± SE) sterile broth = 4.90 broods (± 0.25), dead bacteria = 4.89 broods (± 0.38), live bacteria = 4.15 broods (± 0.26); $F_{2,96} = 2.69$, $P = 0.073$]. We looked in more detail at the success of each breeding attempt by females in the three treatments. We considered the effects of brood and treatment on the number, mean weight and total weight of larvae produced. In each case, there was a significant interaction between brood and treatment. After controlling for the effect of carcass weight ($F_{1,460} = 96.67$, $P < 0.001$), the number of larvae that survived to disperse tended to decrease with each successive brood, but this decrease was significantly more marked in females that initially bred on both live and dead bacteria-dipped carcasses than those in the control treatment (brood × treatment: $F_{8,380} = 3.13$, $P = 0.002$; Fig 3a). After controlling for carcass weight ($F_{1,456} = 175.63$, $P < 0.001$), mean brood mass declined rapidly with each successive brood in the dead bacteria treatment, but mean weight only declined marginally in the live bacteria treatment (brood × treatment: $F_{8,377} = 3.19$, $P = 0.002$; Fig 3b). The combined effect of these two factors meant that total brood weight declined with each brood, with the strongest decline being in the dead bacteria treatment and the weakest decline in the sterile controls, with the live bacteria treatment showing an intermediate reduction in total brood mass (brood × treatment: $F_{8,380} = 3.39$, $P < 0.001$; Fig. 3c), even after controlling for carcass weight ($F_{1,456} = 233.48$, $P < 0.001$). In each case, the r^2 from the minimum adequate model was higher than for the alternate models (Table 1).

There was no significant difference in any of the measures of reproductive investment in the first two broods, where females were exposed to treated carcasses (number of larvae: $F_{2,96} = 0.25$, $P = 0.78$; mean larval mass: $F_{2,96} = 0.20$, $P = 0.82$; total larval mass: $F_{2,96} = 1.77$, $P = 0.18$) and overall, females from either of the bacteria-dipped carcass treatments had significantly lower lifetime reproductive success (LRS) than control females, producing fewer offspring during their lives [mean (± SE) sterile broth = 85.3 larvae (± 2.7), live bacteria = 73.0 larvae (± 3.84), dead bacteria = 70.2 larvae (± 7.2); $t_{1,74} > 2.63$, $P < 0.01$], of lower average mass [mean (± SE) sterile broth = 0.1310 g (± 0.0037), live bacteria = 0.1144 g

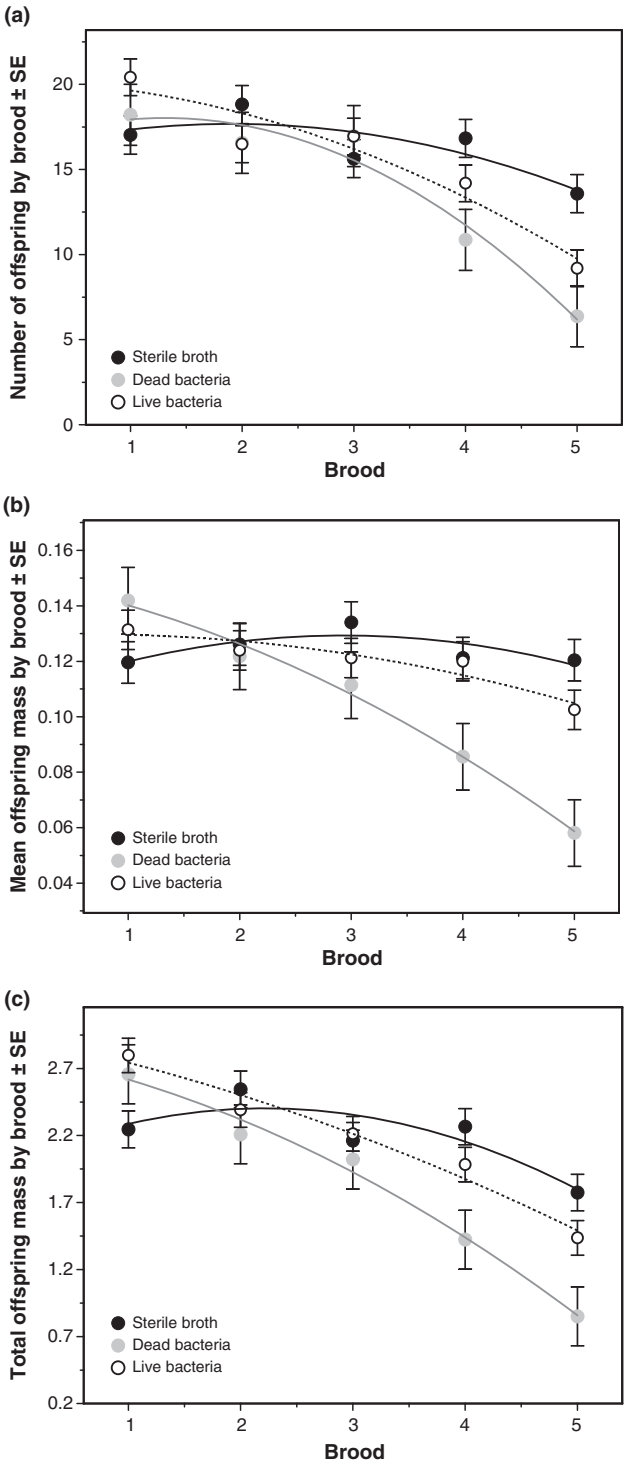

Figure 3 Experiment 3: (a) number of offspring, (b) average offspring mass and (c) total offspring mass per brood produced by females that had been bred continuously throughout their lives. Females were bred twice on a carcass that had either been dipped in nutrient broth (broth) or dipped in a solution of heat-killed (dead bacteria) or live *Micrococcus lysodeikticus* cells in nutrient broth (live bacteria), subsequent carcasses were untreated. All values displayed are estimates from the minimum adequate model controlling for the weight of the mouse carcass that females were provided with and for the random family and individual ID effects.

Table 1 Experiment 3: Estimates of goodness-of-fit of the models of lifetime reproductive success for beetles that had been bred twice on a carcass that had either dipped in nutrient broth or dipped in a solution of dead or live *Micrococcus lysodeikticus* cells in nutrient broth, subsequent carcasses were untreated

Response	Terms included	r^2
Number of offspring	**Carcass weight + brood × treatment**	**0.38**
	Carcass weight + brood + treatment	0.37
	Carcass weight + brood	0.36
Mean larval mass	**Carcass weight + brood × treatment**	**0.39**
	Carcass weight + brood + treatment	0.37
	Carcass weight + brood	0.35
Total larval mass	**Carcass weight + brood × treatment**	**0.53**
	Carcass weight + brood + treatment	0.51
	Carcass weight + brood	0.50

The minimum adequate model in each case and its corresponding r^2 value are highlighted in bold.

(± 0.0063), dead bacteria = 0.1059 g (± 0.0082); $t_{1,74} > 2.27$, $P < 0.026$], resulting in a lower total brood mass [mean (± SE) sterile broth = 11.74 g (± 0.35), live bacteria = 10.02 g (± 0.50), dead bacteria = 9.38 g (± 0.88); $t_{1,74} > 2.82$, $P < 0.0062$]. There was no significant difference between the two bacteria treatments in any measure of LRS ($t_{1,74} < 0.828$, $P > 0.41$).

DISCUSSION

By forcing breeding female burying beetles to upregulate antibacterial activity in their anal exudates, we uncovered substantial lifetime fitness costs associated with mounting this form of social immune response (Fig. 3). Females induced to produce exudates with greater levels of antibacterial activity reared, on average, 14 fewer offspring during their lives than control females, representing a 16% decrease in lifetime reproductive output. To our knowledge, this the first evidence that mounting an immune response of any sort (i.e. social or personal) bears associated costs that reduce lifetime reproductive success, independent of any costs imposed by a parasite.

For females breeding on bacterially challenged carcasses, their reduced lifetime reproductive success was the result of an accelerated age-related decline in fecundity coupled with lower rates of survival. Females exposed to live bacteria maintained a similar average larval mass at

dispersal across breeding attempts, but those exposed to dead bacteria showed a rapid decline in larval brood mass. It is not clear why exposure to dead bacteria would result in a stronger response than exposure to live bacteria, but one possibility is that autoclaving the live culture could break up the cells, presenting many more 'pieces' of bacteria, which may give the impression of a larger bacterial dose.

In both bacteria treatment groups, the number of dispersing larvae produced declined rapidly with each successive brood, resulting in a lower total brood mass than in control females. Therefore, either these females laid fewer eggs per brood than control females, or they cannibalized a larger proportion of their hatchlings than controls (Bartlett & Ashworth 1988). We can be confident that the reduced survival rates shown by females that bred initially on bacteria-dipped carcasses (Fig. 2) were not due to any pathogenic effects of the bacteria on the carcass because females exposed to these bacteria in a single breeding attempt did not subsequently die at a faster rate than control females (Experiment 2). Additionally, the survival rates of females exposed to dead bacteria were lower even than those of females exposed to live bacteria, ruling out the possibility that the actively replicating bacteria were compromising the health of females.

It might be argued that the costs we report here are not causally related to mounting an immune response, but instead are correlated with other duties of parental care such as skinning of the carcass, or larval provisioning. However, our experimental results allow us to reject this alternative interpretation of the data. We can be confident that the effort devoted to carcass skinning did not account for the differences between the treatments because we statistically controlled for the weight of the carcass in each of the models. Although there was an effect of carcass size on subsequent female survival, there was an additional independent effect of carcass bacterial treatment on female lifespan. We have previously shown that larval provisioning carries lifetime reproductive costs in these beetles (Ward *et al.* 2009), but our experiments show that lytic activity in the anal exudates is upregulated independently of larval provisioning. Beetles with higher levels of lytic activity in their anal exudates did not provide their offspring with more food, because brood mass, a key indicator of parental provisioning (e.g. Rozen *et al.* 2008), did not differ between the treatments on the manipulated carcasses (Experiments 2 and 3). Consequently, the lifetime reproductive costs exposed in Experiment 3 must be attributable to the upregulation of lytic activity in the anal exudates. Therefore, our experiments here, and elsewhere (Ward *et al.* 2009), show for the first time that different components of parental care, namely antibacterial carcass defence and larval provisioning, each carry independent lifetime reproductive fitness costs.

The substantial fitness costs associated with mounting a social immune response explain why beetles upregulate the lytic activity in their exudates only when it is required, namely during carcass preparation and brood care (Cotter & Kilner 2010b). These costs also account for the additional fine-tuned plasticity in antibacterial production that we found in response to a bacterial challenge to the carcass (Fig. 1). Presumably, by carefully modulating production of antibacterial substances in their anal exudates in relation to the extent of bacterial challenge, female beetles can minimize the fitness costs of mounting a social immune response. The social immune system in the burying beetles thus appears to be deployed in a relatively sophisticated way, and is seemingly just as plastic as the personal immune response (see Wilson & Cotter 2009 and references therein). What cues might females use for adjusting investment in antibacterial activity in their anal exudates? In our previous work, we found that lytic levels in the anal exudates decrease rapidly once beetles are removed from the carcass suggesting that the presence of the carcass alone is sufficient to trigger some antibacterial production (Cotter & Kilner 2010b). The experiments we describe here suggest that the bacteria themselves present an additional cue, possibly detected orally as has been shown in cabbage looper caterpillars (Freitak *et al.* 2007). However, we have not yet determined whether beetles are responding to the presence of bacteria alone, or to specific concentrations of bacteria on a carcass. It is worth noting that broth alone also appears to upregulate antibacterial activity, though to a lesser extent than the bacterial treatments (Fig. 1). This may be because, whilst the broth is sterile, once it comes into contact with the carcass it may encourage the replication of bacteria already present, thereby presenting a heavier dose than the untreated carcasses, though still less than the bacteria-treated carcasses, suggesting that there may indeed be dose response to bacterial contamination.

The usage costs (*sensu* Schulenburg *et al.* 2009) of the social immune system that we report here could arise simply through resource re-allocation (Sheldon & Verhulst 1996). Upregulating antibacterial activity in the anal exudates might cause females to divert resources otherwise destined for tissue repair and/or egg production, thereby compromising survival and reducing fecundity. It is likely that lytic activity is competing for specific nutrients or amino acids rather than energy *per se*, due to the fact that survival does not differ under starvation. Hormones could play a key role in mediating this resource allocation; a single hormone can have antagonistic effects on tissues that are competing for resources (Finch & Rose 1995). Juvenile hormone (JH) is one such candidate hormone in the burying beetle (Trumbo & Robinson 2004). In a congeneric burying beetle to our study species, *N. orbicollis*, JH levels are upregulated following the discovery of a carcass, and this JH surge

appears to initiate ovarian development and caring behaviour (Trumbo *et al.* 1995; Scott & Panaitof 2004). It is likely that this hormonal surge also initiates the upregulation of antibacterial activity, along with the other parental care behaviours (Cotter & Kilner 2010b), and the downregulation of PO, as has been shown in insect haemolymph (Rolff & Siva-Jothy 2002; Rantala *et al.* 2003). Separate experiments have established that elevated JH levels also reduce adult survival, at least when food is restricted, potentially via increasing the rate of metabolism (Trumbo & Robinson 2004). Thus, it is possible that increased JH levels may result in the channelling of resources to reproduction, including the social immune response, and away from somatic maintenance, although further experiments are necessary to test whether this is the case or not.

Finally, defending a public resource brings fitness benefits to other individuals (Rozen *et al.* 2008) but we have shown that this comes at some personal costs to females (Fig. 3). Therefore, mounting a social immune response in the burying beetle is cooperative (Hamilton 1964; West *et al.* 2007). In its narrowest sense, this form of cooperation (West *et al.* 2007) is just like any other sort of parental investment, with females sacrificing their future fitness to assist their offspring. Similarly, the female's investment in social immunity benefits her partner too because presumably he then escapes some of the costs of defending the carcass from bacteria. Exactly how these costs are divided between the breeding pair will be a source of sexual conflict (Lessells 1999) and our experiments to date suggest that females are on the losing side. Male anal exudates typically exhibit less antibacterial activity than those of their partners (Cotter & Kilner 2010b), and males also have greater lifetime reproductive success, when all else is equal between the sexes (Ward *et al.* 2009). Whether these two observations are causally linked, and whether females can ever pass the costs of social immune defence onto her partner (Chase 1980; Houston & Davies 1985; McNamara *et al.* 1999) remain to be determined in future work.

In nature, a carcass cannot always be monopolized by a single breeding pair and uneasy breeding associations between three or more adults commonly arise (Müller *et al.* 2007). How should the costs of social immunity then be divided amongst the breeding adults (who are unrelated)? If all adults arrive at the carcass at the same time, then the problem resembles a public goods dilemma, in which each individual participates to achieve a common good but at some cost to themselves (Dionisio & Gordo 2006; Frank 2010). Theoretical analyses suggest that where the common good is not diminishable (Dionisio & Gordo 2006), or where the group is dependent on the common good (Frank 2010), (as in this case), then the evolutionary stable strategy is for everyone to make some contribution. But adults do not always arrive at the carcass simultaneously (Eggert & Müller 1997). Takeovers of prepared carcasses are common (e.g. Trumbo 1990), presumably because the victorious new owners thereby avoid some of the fitness costs associated with defending the carcass against bacterial rivals. Even in partial takeovers, when carcass owners cede some part of their breeding resource to another adult (Trumbo 1990; Eggert & Müller 1992), the incoming breeder presumably benefits from the prior investment in social immune defence by the resident beetles.

As well as potentially benefiting unrelated conspecifics, a female's investment in social immunity can also potentially be of value to the wider carrion community, particularly the phoretic nematodes (Richter 1993) and mites (Brown & Wilson 1992; Schwarz & Koulianos 1998) who breed alongside the beetle's larvae on the carcass and who would presumably gain from the beetle's antibacterial defences of the resource. Previous work on other species of burying beetle has established that phoretic mites might themselves also make a contribution to social immunity by foraging on any nematodes and microbes that take up residence on the carcass (Wilson & Knollenberg 1987; see also Biani *et al.* 2009). It would be interesting to determine in future work whether the beetles and the phoretic community make complementary contributions to social immunity, together providing a more robust defence of the carcass than they would achieve alone, or whether the phoretic community benefits the burying beetle directly by enabling it to reduce investment in social immune defences.

In conclusion, our experiments show that mounting a social immune response has major fitness costs for females: females that upregulate their antibacterial activity produce only 84% of the offspring that control females manage to raise during their lives. The challenge for future work is to identify the mechanisms responsible for these costs and to determine how investment in social immunity is shared within burying beetle pairs, and breeding associations on a carcass, as well as amongst the wider carrion community.

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