Title: Parental care influences social immunity in burying beetle larvae

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Running title: Social immunity in burying beetle larvae

Abstract

1. We provide evidence for social immunity in offspring of a sub-social species, the burying beetle, *Nicrophorus vespilloides.*

2. *N. vespilloides* is a carrion breeder, and in a similar fashion to the adult beetles, the offspring produce exudates that exhibit lytic activity, which are used to coat the breeding resource. This strategy defends against the microbial community.

3. The lytic activity in larval exudates declines as the brood develops, perhaps being most beneficial at the start of the breeding bout.

4. Changing levels of parental care through widowing/orphaning affects lytic activity in the larval exudates, with levels decreasing in the absence of both parents.

Introduction

The burying beetle, *N. vespilloides* (Figure S1, Supplementary Material), breeds on the carcass of small mammals and exhibits bi-parental care, one component of which is a social immune response (Cotter & Kilner 2010b). Cotter & Kilner suggest that "any type of immune response that has been selected to increase the fitness of the challenged individual and one or more recipients should be classified as social immunity" (2010b). The beetles coat the carcass with antimicrobial anal exudates (Cotter & Kilner 2010a) in order to minimise competition from the microbial community, delaying decomposition of the carcass (Rozen et al. 2008). This social immune response is costly; antibacterial activity is only upregulated in the presence of a carcass (Cotter & Kilner 2010a) and forced upregulation reduces lifetime reproductive success (Cotter et al. 2010). In *N. vespilloides*, both sexes produce exudates, however, the females' exudates show higher levels of antibacterial activity

than the males (Cotter & Kilner 2010a). The beetles can also flexibly adjust the level of antibacterial activity in response to mate loss.

Observations have shown that the larvae also produce exudates throughout their development, though their potential antibacterial activity is unknown. With production of these exudates being so costly to the parents, it would benefit them if their offspring were also able to partake in carcass preservation. Furthermore, we know that larvae of this species can survive without parental care, albeit at a reduced rate and with a resulting poorer quality (Eggert et al. 1998). Their ability to survive the loss of both parents may be due in part to the production of antibacterial substances. Here we ask whether 1) antibacterial activity is present, 2) how it changes during larval development and 3) whether larvae, like adults, can flexibly alter levels of antibacterial activity in response to changing conditions, for example, presence/absence of parents.

Materials and Methods

N. vespilloides colony

The colony was established from a pedigreed *N. vespilloides* colony at the Department of Zoology, University of Cambridge. Beetles were maintained as described previously (Cotter & Kilner 2010a).

Experiment 1 – characterising antibacterial activity across the larval stage

Exudates were collected from all larvae in a brood using a capillary tube and pooled in a single eppendorf tube. This enabled us to use a known volume of exudate in the later analysis (1 μl). Larvae were sampled from day 1 (hatching) to day 5 (dispersal). Day 1 and 2 larvae were so small that exudate collection with a capillary tube was not possible. Instead, the tip of the abdomen was gently pressed against a single punched circle of filter paper for

every larva in the brood. Whilst volume was impossible to control for in these samples, we collected <1 μl of exudate sample on each filter paper circle, and so the level of lytic activity would be a slight under-estimate compared to day 3, 4 and 5 larvae. All samples were stored at -20°C until testing. Larvae were sampled from fifty-five families, though some were sampled on more than one day, giving 75 samples in total. Forty-two families were sampled from once, nine families were sampled from twice and five families were sampled from three times. The numbers of larvae from which samples were collected and pooled on each day were as follows: day 1 = 10.33 larvae +/-2.01, day 2 = 14.63 larvae +/- 1.49, day 3 = 9.65 larvae +/-1.24, day 4 = 14.27 larvae +/-1.88 and day 5 = 7.17 larvae +/- 2.29.

Experiment 2 – the effect of reduced parental care on larval antibacterial activity

Sixty pairs were established and were assigned to one of four treatment groups, male removed, female removed, both removed, or neither removed (control). On the day of hatching, parents were removed according to the treatment and the larval exudate sampled daily as described above. Exudate collection was more successful for this experiment, sixty families were sampled daily for five days giving 300 samples in total. Breeding success was recorded for all families.

Antibacterial activity

Lysozyme-like antibacterial activity was measured as described previously (Cotter & Kilner 2010a). In brief, 1 μl of each sample was pipetted into a hole in an agar plate inoculated with freeze-dried *Micrococcus lysodeikticus* cells and incubated for 24 hours at 33°C. We selected *Micrococcus lysodeikticus* as it is a soil bacterium, which is the breeding environment of the burying beetle. It is one of the microorganisms analysed in Hall et al. (2011) and in that study was inhibited by secretions from *Nicrophorus*. It is also the main bacteria used for lytic plates in studies on burying beetles (Cotter et al. 2010, Steiger et al. 2011, Arce et al. 2012) and many other insect species e.g. locusts (Wilson et al. 2002). Filter

paper circles were placed directly onto the surface of the agar. Clear zones in the agar were measured with digital calipers and calibrated against a lysozyme standard (Figure S2, Supplementary Material). In order to test for the potential mechanical differences between using filter paper and punched holes, we compared samples tested using both methods and found no difference between the size of the clear zone produced by 1 μl of sample pipetted into a hole and that produced using filter paper placed on the surface of the agar ($F_{1,25} = 0.27$, $P = 0.61$). We were therefore able to reliably compare samples measured using the different techniques. Whilst other methods are now available to measure antimicrobial activity, the majority of analyses to date in this and many other insect species have been carried out using zones of inhibition on agar plates. Using this method allows us to compare across studies to some extent.

Statistical analyses

Antibacterial activity was log-transformed to approximate normality. Lytic data from both experiments was analysed with Restricted Estimate Maximum Likelihood (REML) models in Genstat 15 (VSN International, Hemel Hempstead, UK) with family included as a random effect to account for multiple testing from each family. Breeding data were analysed using a generalised linear model (GLM). Carcass weight and interaction terms were included in all models but were removed due to non-significance. Figures were produced in R 2.15.1 (Development Core Team, 2013).

Results and Discussion

We show for the first time in this study that the larval exudates contain antibacterial substances. It has been shown previously that insect larvae have the ability to produce antibacterial secretions e.g. blowfly larvae, *L. sericata* (Kerridge et al. 2005), however this strategy has not been widely documented. Levels of lytic activity were highest in the newly-

hatched larvae and declined throughout the brood (REML: $F_{1,49}$= 51.27, P < 0.001; Figure 1). Indeed, as the exudate volume collected from day 1 and day 2 larvae was < 1 μl, the lytic activity at this stage is actually slightly underestimated, and so in reality should be even higher than observed. If the trend had been in the other direction i.e. larval exudate activity increasing with age, we could not have drawn reliable conclusions as in this case a lower activity in day 1 and day 2 larvae could have been due to a smaller exudate volume. As we did not collect a fixed amount of exudate from day 1 and 2 larvae (although volume < 1 μl), we tested whether activity was dependent on the number of larvae from which it was collected, however there was no effect (REML: $F_{1,27}$ = 0.51, P = 0.48). The pattern of decline in larval lytic activity mirrors that of the parents; highest when larvae arrive on the carcass (Cotter et al. 2013). The need for lytic exudates may fall after a high amount of initial preservation, resulting in increasing sterility. For the parents, assistance from the larvae may mean that they do not need to invest as heavily in lytic activity, which could promote further reproductive success and longevity.

With regards parental removal experiments, preliminary data exploration showed that the effects of removing males and females were very similar but different from the other two treatment groups. Therefore we grouped these two treatments into a single treatment representing "one parent removed". Lytic activity was much lower in larvae where both parents were removed (REML: $F_{2,46}$= 3.66, P = 0.033; Figure 2a) and there was a significant effect of brood age, with the pattern being very similar to experiment 1 (REML: $F_{1,210}$= 119.95, P < 0.001). These results illustrate that, like the parents (Cotter & Kilner 2010a), the larvae exhibit plasticity with regards lytic activity levels. Whilst we hypothesised that this antibacterial activity may contribute to their survival in orphaned conditions, activity actually decreased. This drop may be due to the fact that the larvae must now invest in other activities, for example self-feeding, at the detriment to lytic investment. Their condition is

also likely to be compromised even at this stage, which may cause a decline in lytic activity. A different experimental set-up may be required to unveil an upregulation of lytic activity in the larvae, for example using a rotten carcass but maintaining parental assistance i.e. a greater requirement for combatting microorganisms but without the strain of self-feeding. The number of larvae produced was lower when both parents were removed post-hatching (GLM: $F_{2,51}$= 4.30, P = 0.019; Figure 2b), but the mean weight of larvae was not affected (GLM: $F_{2,49}$ = 2.07, P = 0.137). This is consistent with findings from previous studies (Rozen et al. 2008; Arce et al. 2012).

Future experiments should consider the cost of lytic activity from the larval perspective. The larvae may be constrained developmentally if the mechanism for production in the parents also drives production in the larval stage. In light of plasticity from parents and their offspring, future studies should consider the scope for conflict. For example, if the larvae didn't produce antibacterial exudates, would the parents be forced to produce more? To conclude, we show that larvae produce antibacterial exudates and that parental care influences the extent of social immunity.

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Figure legends

Figure 1

The decline in the antibacterial activity of larval exudate over time. Larvae hatch on day 1 and disperse from the carcass on day 5.

Figure 2

The effect of removing one or both parents on a) the antibacterial activity of larval exudate and b) the number of larvae dispersing from the carcass. Means and SEs in a) are predicted values from a REML model controlling for family.

Figures

Figure 1

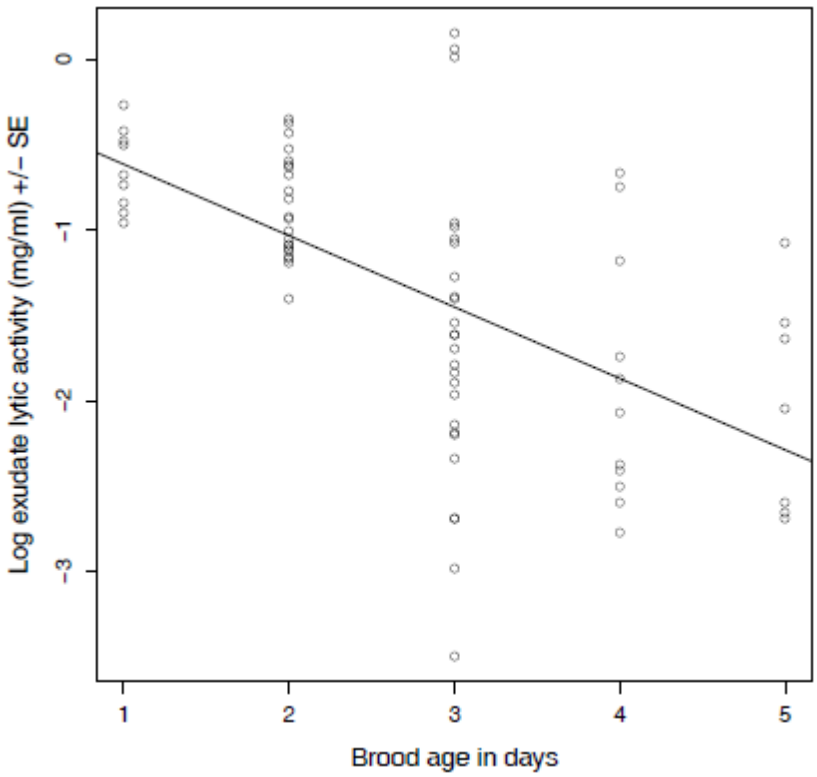
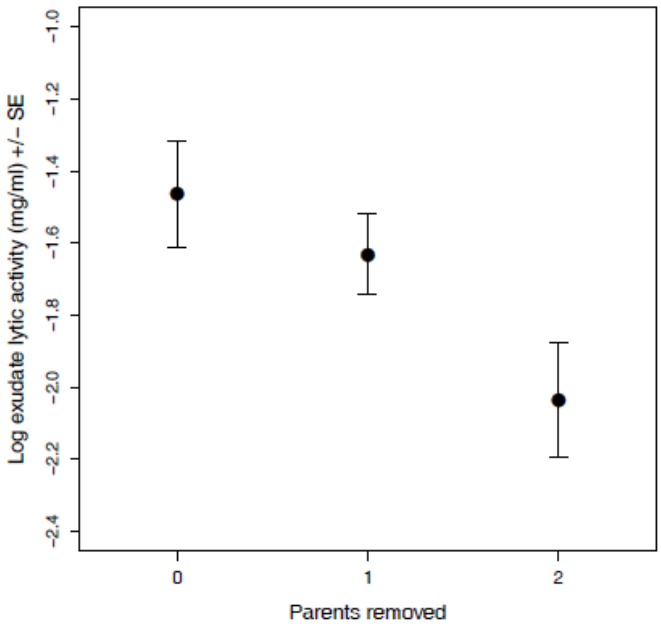
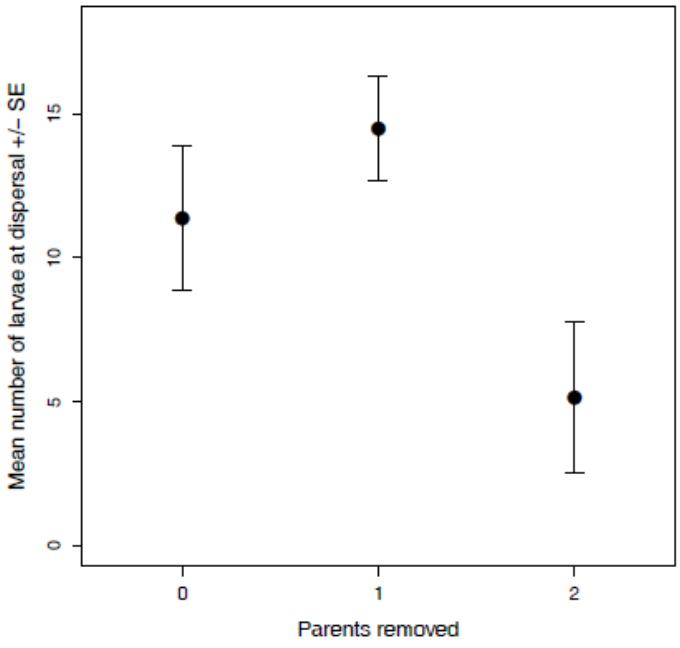


Figure 2

a)


Log exudate lytic activity (mg/ml) +/- SE
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
-2.2
-2.4
0
1
2
Parents removed

b)


Mean number of larvae at dispersal +/- SE
0
5
10
15
0
1
2
Parents removed