Ecological Entomology

Ecological Entomology (2014), DOI: 10.1111/een.12099

SHORT COMMUNICATION

Parental care influences social immunity in burying beetle larvae

CATHERINE E. REAVEY, LAURA BEARE and SHEENA C. COTTER School of Biological Sciences, Queen's University Belfast, Belfast, U.K.

Abstract. 1. In this study, evidence is provided of social immunity in the offspring of a sub-social species, the burying beetle, *Nicrophorus vespilloides*.

2. *Nicrophorus 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.

Key words. Antibacterial, ecological immunology, insect, lysozyme, *Nicrophorus*, parental care, social immunity.

Introduction

The burying beetle, *Nicrophorus vespilloides* (Figure S1), breeds on the carcass of small mammals and exhibits biparental care, one component of which is a social immune response (Cotter & Kilner, 2010b). Cotter and Kilner (2010b) 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'. 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, although 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.

Correspondence: Catherine E. Reavey, School of Biological Sciences, Queen's University Belfast, MBC, 97 Lisburn Road, Belfast BT9 7BL, U.K. E-mail: creavey01@qub.ac.uk

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: (i) whether antibacterial activity is present; (ii) how it changes during larval development; and (iii) whether larvae, like adults, can flexibly alter levels of antibacterial activity in response to changing conditions (e.g. presence/absence of parents).

Materials and methods

Nicrophorus 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 $< 1\,\mu l$ of exudate sample on each filter paper circle, and so the level of lytic activity would be a slight underestimate compared with day 3, 4 and 5 larvae. All samples were stored at $-20\,^{\circ}C$ until testing. Larvae were sampled from 25 families, although some were sampled on more than one day, giving 75 samples in total. Forty-two families were sampled once, nine families were sampled 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 $\pm$ 2.01; day 2, 14.63 larvae $\pm$ 1.49; day 3, 9.65 larvae $\pm$ 1.24; day 4, 14.27 larvae $\pm$ 1.88; and day 5, 7.17 larvae $\pm$ 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 was sampled daily, as described in the previous section. Exudate collection was more successful for this experiment; 60 families were sampled daily for 5 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, $1\,\mu l$ of each sample was pipetted into a hole in an agar plate inoculated with freeze-dried *Micrococcus lysodeikticus* cells and incubated for 24 h at $33\,^{\circ}C$. We selected *M. 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 on to the surface of the agar. Clear zones in the agar were measured with digital calipers and calibrated against a lysozyme standard (Figure S2). 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 $1\,\mu l$ 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.

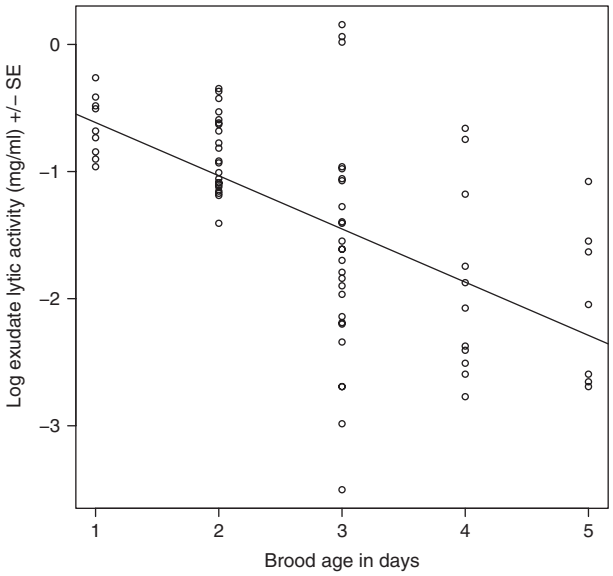

Fig. 1. The decline in the antibacterial activity of larval exudate over time. Larvae hatch on day 1 and disperse from the carcass on day 5.

Statistical analyses

Antibacterial activity was log-transformed to approximate normality. Lytic data from both experiments were analysed using 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 (R 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 previously been shown that insect larvae have the ability to produce antibacterial secretions (e.g. blowfly larvae, *Lucilia sericata*; Kerridge *et al.*, 2005); however, this strategy has not been widely documented. Levels of lytic activity were at their highest in the newly hatched larvae and declined throughout the brood (REML: $F_{1,49} = 51.27$, $P < 0.001$; Fig. 1). Indeed, as the exudate volume collected from day 1 and day 2 larvae was $< 1\,\mu l$, 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 day 2 larvae (although volume was $< 1\,\mu l$), we tested whether activity was dependent on the number of larvae from which it was collected; however there was no effect (REML: $F_{1,27} = 0.51$, $P = 0.48$).

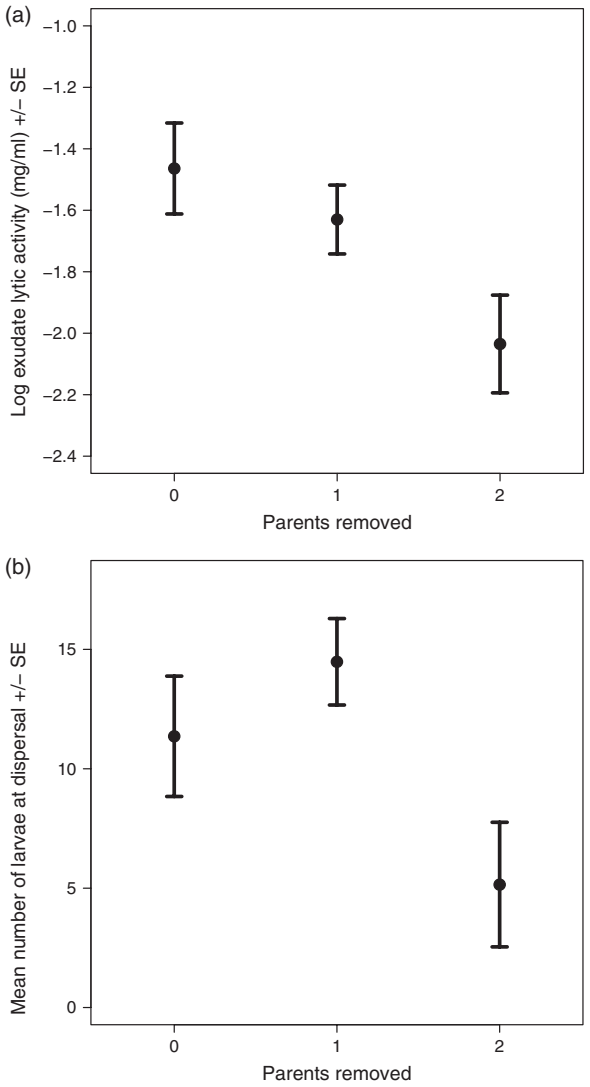

Fig. 2. The effect of removing one or both parents on the antibacterial activity of larval exudate (a) and the number of larvae dispersing from the carcass (b). Means and SEs in (a) are predicted values from a restricted estimate maximum likelihood model, controlling for family.

The pattern of decline in larval lytic activity mirrors that of the parents – at its 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 regard to 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 in cases where both parents were removed (REML: $F_{2,46} = 3.66$, $P = 0.033$; Fig. 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 regard to lytic activity levels. Whilst we hypothesised that this antibacterial activity could 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 (e.g. self-feeding) to the detriment of 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 setup may be required to reveal an upregulation of lytic activity in the larvae, for example, using a rotten carcass but maintaining parental assistance, i.e. a greater requirement for combating microorganisms but without the strain of self-feeding. The number of larvae produced was lower when both parents were removed post-hatching (GLM: $F_{2,51} = 4.30$, $P = 0.019$; Fig. 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 the plasticity from parents and their offspring, future studies should consider the scope for conflict. For example, if the larvae did not produce antibacterial exudates, would the parents be forced to produce more? To conclude, we have shown that larvae produce antibacterial exudates and that parental care influences the extent of social immunity.

Acknowledgements

C.E.R. was supported by a DEL studentship, and S.C.C. was supported by a NERC fellowship (NE/H014225/2).

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12099

Figure S1. A *Nicrophorus vespilloides* burying beetle providing care for her brood. Photo courtesy of O. Kruger.

Figure S2. An example of the lytic zone of inhibition assay. The plates are prepared by using 10 ml of 1% agar with 5 mg per ml freeze-dried *M. lysodeikticus*. 2 mm diameter holes are punched in each plate and 1 μl of exudate placed in each well. Following incubation at 33 °C for 24 h the diameter of the zone of inhibition is measured and lytic activity determined by comparison with a serial dilution of hen egg white lysozyme.

References

Arce, A.N., Johnston, P.R., Smiseth, P.T. & Rozen, D.E. (2012) Mechanisms and fitness effects of antibacterial defences in a carrion beetle. *Journal of Evolutionary Biology*, **25**, 930–937.

Cotter, S.C. & Kilner, R.M. (2010a) Sexual division of antibacterial resource defence in breeding burying beetles, *Nicrophorus vespilloides*. *Journal of Animal Ecology*, **79**, 35–43.

Cotter, S. & Kilner, R. (2010b) Personal immunity versus social immunity. *Behavioral Ecology*, **21**, 663–668.

Cotter, S., Topham, E., Price, A. & Kilner, R. (2010) Fitness costs associated with mounting a social immune response. *Ecology Letters*, **13**, 1114–1123.

Cotter, S.C., Littlefair, J.E., Grantham, P.J. & Kilner, R.M. (2013) A direct physiological trade-off between personal and social immunity. *Journal of Animal Ecology*, **84**, 846–853.

Eggert, A.K., Reinking, M. & Müller, J.K. (1998) Parental care improves offspring survival and growth in burying beetles. *Animal Behaviour*, **55**, 97–107.

Hall, C.L., Wadsworth, N.K., Howard, D.R., Jennings, E.M., Farrell, L.D., Magnuson, T.S. *et al.* (2011) Inhibition of Microorganisms on a Carrion Breeding Resource: The Antimicrobial Peptide Activity of Burying Beetle (Coleoptera: Silphidae) Oral and Anal Secretions. *Environmental Entomology*, **40**, 669–678.

Kerridge, A., Lappin-Scott, H. & Stevens, J. (2005) Antibacterial properties of larval secretions of the blowfly, *Lucilia sericata*. *Medical and Veterinary Entomology*, **19**, 333–337.

R Development Core Team (2013) R: *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. ISBN 3-900051-07-0 [WWW document]. URL http://www.R-project.org [accessed on 1 May 2013].

Rozen, D.E., Engelmoer, D.J.P. & Smiseth, P.T. (2008) Antimicrobial strategies in burying beetles breeding on carrion. *Proceedings of the National Academy of Sciences*, **105**, 17890–17895.

Steiger, S., Gershman, S.N., Pettinger, A.M., Eggert, A-K. & Sakaluk, S.K. (2011) Sex differences in immunity and rapid upregulation of immune defence during parental care in the burying beetle, *Nicrophorus orbicollis*. *Functional Ecology*, **25**, 1368–1378.

Wilson, K., Thomas, M.B., Blanford, S., Doggett, M., Simpson, S.J. & Moore, S.L. (2002) Coping with crowds: Density-dependent disease resistance in desert locusts. *Proceedings of the National Academy of Sciences*, **99**, 5471–5475.

Accepted 6 December 2013