Title: Sex-specific signals influence intraspecific aggregation in the temperate dung beetle *Aphodius fossor*.

Authors: Paul Manning[1,2], Jack Ford[3]

1. Department of Zoology, University of Oxford, Oxford, U.K.
2. Dr Beynon's Bug Farm, St Davids, U.K.
3. School of Biosciences, Cardiff University, Cardiff, U.K.

Abstract:

1. In temperate climates dung is often colonised by several species of endocoprid (dwelling) dung beetle which use pats for feeding, mating, and reproduction.
2. Beetles aggregate even when offered patches (dung pats) of consistent age, size, and origin, suggesting that beetles themselves might influence the attractiveness of the patches they colonise to members of their own species. Both pheromones and physical changes to the structure of dung pats caused by colonising beetles have been suggested as mechanisms facilitating intraspecific aggregation, but neither of these hypotheses has been tested empirically.
3. Using a common European dung beetle (*Aphodius fossor*), we conducted a simple choice experiment designed to test whether 1) earlier colonisation by conspecifics could alter dung attractiveness, and 2) whether attraction was influenced by sex-specific signals.
4. We show that female beetles are repelled by dung colonised by conspecific females, and are attracted to dung colonised by conspecific males. Male beetles show no evidence of attraction or repellence for dung colonised by either sex. In neither case was uncolonised dung found to be less attractive than predictions of non-preference.

5. Our results suggest that sex-specific signals and responses may help explain aggregated dung beetle distributions.

Introduction

Dung beetles (Coleoptera: Scarabaeidae) are a popular model taxon for testing ecological theory, including biodiversity-ecosystem functioning relationships (Beynon *et al.*, 2012), metapopulation dynamics (Roslin, 2000; Roslin & Koivunen, 2001), and species-area relationships (Lobo & Martín-Piera, 1999). The spatial ecology of these beetles has been studied extensively, as habitat patches (dung pats) are easily delimited and species distributions can be studied readily over a wide range of scales (e.g. Finn *et al.*, 1998).

In contrast to the intense competition experienced amongst and within communities of tropical dung beetles, in temperate ecosystems competition is relatively weak and dung monopolisation rarely occurs (for review see Finn & Gittings, 2003). Instead, dung beetle communities tend to be dominated by dwelling beetles, with density and diversity showing an aggregated distribution across habitat patches (Palestrini *et al.*, 1998).

An important element that influences dung beetles aggregation within a localised patch is dung attractiveness. This can be influenced by a variety of factors including time of day at which the dung was produced (Holter, 1979), exposure of the dung pat (Landin, 1961), weather during dispersal events (Finn *et al.,* 1998), age of the dung (Landin, 1961), and presence of veterinary pharmaceutical residues (Floate, 2007). However, highly variable beetle aggregation is observed even when using dung from a homogenous source with patches having both identical size and age (Hanski & Cambefort, 1991; Palestrini *et al.,* 1998). This suggests an additional, unidentified mechanism may be promoting aggregation within dung pats.

A study exploring spatial distribution of temperate dung beetles (Palestrini *et al.* 1998) found both inter and intraspecific aggregation, and proposed that activity of early colonising beetles might increase attractiveness and habitability of dung for potential future colonisers. A second hypothesis offered in discussion within the same paper, suggested that pheromones might be contributing to intraspecific aggregation.

We conducted a preliminary investigation of these hypotheses with a choice experiment, using the widely-distributed temperate dung beetle *Aphodius fossor* L. We offered male and female beetles choice of three dung pats which were either: uncolonised, or manipulated to contain three conspecific males or three conspecific females. By comparing observed frequencies with expectations of non-preference, we were able to test whether colonisation patterns supported aggregations promoted by (1) enhanced attractiveness due to activity of colonised beetles, or (2) sex-specific responses.

Methods

Collection of dung beetles

Adult *A. fossor* were hand-collected from cattle dung at Dr Beynon's Bug Farm, St Davids, Pembrokeshire, United Kingdom (51°53′20″, 5°14′09) on May 30th 2015. Adults were separated by sex into well-ventilated 4 L plastic tubs, with damp playground sand and 50 mL of fresh cattle dung. All beetles were stored in a cool, dark shed before beginning the experiment.

Experimental set-up

Freshly-deposited dung was collected before any invertebrates were able to colonise from a group of Welsh Black cattle at the same farm. The dung was homogenised, and nine 250 mL dung pats were formed. Each dung pat was placed into a well-ventilated, 4 L rectangular tub (22 cm × 15 cm × 15 cm), on top of 8 cm of moist, washed playground sand. Four female beetles (n=3) or four male beetles (n=3) were randomly assigned to tubs. The three remaining dung pats were left uncolonised to serve as controls. Tubs were stored for 48 hours in a cool, dark shed to allow beetles to acclimatise before starting the experiment.

Three arenas were constructed using circular, 30 cm diameter, 14 L black plastic plant pots filled to 10 cm with moist, washed playground sand (Figure 1). Three circular holes measuring 2 cm in diameter were drilled at intervals of 120^0, the bottom of the hole sitting flush with the sand surface. A 4 cm length of clear plastic tubing (2 cm diameter) was used to join the larger arena to the tubs containing the dung pats. The innermost end of the tube was placed flush against the side of the arena, and extended into the smaller tubs (Fig 1).

Dung choice tests

On June 1st at 19:00h, two hours before the experiment began, the tubs containing dung pats were attached to the larger arena. The first round of the experiment began at 21:00h when *A. fossor* becomes most active (P. Manning, pers. obs.). A total of 90 males and 90 females were run through the experiment for a total of 18 separate rounds (occurring in six concurrent periods). Sand was re-moistened with water between rounds, using a spray bottle. The experiment was conducted indoors, under fluorescent light. Arenas and tubs were rotated 120^0 following each round to account for any differences caused by differences in ambient light. We ran male beetles through the experiment first, replacing the top c. 3 cm of sand within the arena before testing female beetles.

Three arenas were run simultaneously. As pilot trials found that beetles frequently took longer than twenty minutes to select a dung pat, beetles were run in groups of ten. In each round, the 10 beetles of a single sex were added to the middle of each arena and were initially confined to the innermost area of the arena using a circular, steel tea strainer (8 cm diameter). Beetles were kept in place for 120 seconds before the tea strainer was lifted and beetles were able to move towards a dung pat in one of the surrounding tubs. Each round of the experiment continued until either all beetles had reached a tub (each beetle being promptly captured and removed from the experiment before it could enter the dung) or after 25 minutes, when the round was terminated.

Analysis

Data for male and female beetles were analysed separately using a goodness-of-fit test for discrete multivariate data, comparing observations to null expectations of non-preference. When observed counts were found to be significantly different from null expectations ($P < 0.05$), a post-hoc test was performed, testing each category of observation against its expected frequency (McDonald, 2009) using a Bonferroni corrected significance threshold ($\alpha = 0.0167$). Analyses were carried out using the "EMT" (Menzel, 2013) package for R 3.1.1 (R Core Team, 2014).

Results and Discussion

Of the 90 females tested, 67 reached the dung within the allotted 25 minutes. The distribution of observed counts of females attempting to colonise dung differed significantly from expectation ($X^2 = 11.32$, df=2, P=0.002). Post-hoc testing suggested that female beetles avoided dung colonised by other females ($X^2 = 10.024$, df=1, $P = 0.001$) while preferentially selecting dung colonised by males ($X^2 = 6.52$, df=1, P=.013) (Figure 2). Of the 90 males tested, 62 reached the dung within the allotted 25 minutes. The majority of male beetles were attracted to dung colonised by females (Figure 3), but the observed values were not significantly different from the expected model of indiscriminate choice ($X^2 = 4.004$, df = 2, P=0.146). In neither case did we find uncolonised dung was selected less frequently than predicted by non-preference, suggesting there was no significant influence of beetle colonisation on dung attractiveness.

The most striking result of our experiment was the avoidance, by newly colonising female beetles, of dung colonised previously by other females. We suggest this might be attributable to deterrent pheromones produced by females, which serve to repel other females, many being classified as ‘anti-aggregation’ or ‘oviposition deterring’ pheromones. These signals discourage crowding and oviposition, thus reducing potential competition for offspring. Production of deterrent pheromones are known from many insects, e.g.: tephritid flies (Prokopy, 1975), weevils (Njihia *et al.*, 2014) and lacewings (Růžička, 2013).We hypothesise that upon receiving a signal indicating the presence of a female beetle, the dispersing female selects an alternative resource where her offspring will be less likely to experience density-dependent mortality. There is strong evidence suggesting that dung beetle larvae can be limited by space within a dung pat (Finn & Gittings, 2003) and, as *A. fossor* is one of the largest species of endocoprid dung beetle in Europe (Jessop, 1986), low-cost strategies (production and reception of chemical signals) could play an important role in reducing density-dependent mortality.

The preference of male-colonised dung by female beetles suggests that males may produce an attraction pheromone. The timing of our study occurred approximately two weeks after teneral adults began emerging, which roughly corresponds to when *A. fossor* females reach reproductive maturity (Gittings & Giller, 1997). The release of attractant pheromones by males could support mate-finding by females in patchy environments (Larsson *et al.*, 2003). In the case of *A. fossor*, beetles are almost exclusively found in coupled pairs, although density may exceed six pairs within a single dung pat (P. Manning, pers. obs.). Attractant pheromones produced by male Scarabaeidae in related species (e.g. Tribe, 1975; Edwards & Aschenborn, 1988; Larsson *et al.*, 2003). Individual males of the temperate species *Typhaeus typhoeus* L. have been observed tunnelling part-way into dung where they defecate on the dung surface (Brussaard, 1983). This behaviour is thought to represent a simultaneous pheromone release. During our experiment, we observed both males and females tunnelling part-way into the dung where they remained for several minutes with their abdomen partially exposed: while we did not observe defecation, this stance may have represented pheromone release.

Alternatively, sex-specific chemical signals influencing dung beetle aggregation may not be pheromone based. The activity of endocoprid dung beetles alters the composition of gases fluxing from cow dung (Penttilä *et al.*, 2013), which might act to modify its attractiveness to colonising beetles. If male or female beetles behave differently within the dung pat (eg: higher activity), this could potentially influence different gas fluxes. As we have no mechanistic evidence for the sex-specific responses, this should not be discounted.

Furthermore, sex-specific signals in dung beetles may not be chemical: acoustic signals have been shown to play a role in aggregation in other *Aphodius* species (Hirschberger, 2001; Kasper & Hirschberger, 2005). However, acoustic signals are unlikely to have contributed to dung choice in this experiment as previous evidence suggests they are effective only at short range, being received mechanically from within the dung pat (Hirschberger, 2001).

As this experiment was conducted only 48 hours after dung was first produced, these data are not fully representative of the entirety that dung beetles remain resident within a dung pat. Adult *A. fossor* often inhabit older dung and it is likely that beetle activity could play a more influential role in altering attractiveness as dung ages and desiccates.

While our experimental design necessitated running beetles in groups of 10 as a consequence of logistical constraints, we recognise that individuals may have been influenced by odours released, and/or signals displayed by other beetles in the group. However, we observed that beetles frequently stopped when reaching the tunnel end, with their head suspended into the airspace above the dung. After a brief (15 – 30s) period of antennal movement, the beetles would either turn, walking back into the arena, or alternatively walk forward – dropping into the tub. This observation suggested that beetles were making a decision in response to cues emanating from the dung pat itself, rather than solely following chemical trails laid by other beetles in the group.

Our results demonstrate that sex-specific attraction could have a strong influence in explaining intraspecific dung beetle aggregations. Experimental work that uses more refined methodologies (eg: y-mazes, electroantennography), and expanding to include additional species would be useful next steps in achieving a better understanding of the underlying mechanisms that influence dung beetle aggregations.

References

Beynon, S.A., Mann, D.J., Slade, E.M. & Lewis, O.T. (2012) Species-rich dung beetle communities buffer ecosystem services in perturbed agro-ecosystems. *Journal of Applied Ecology*, **49**, 1365–1372.

Brussaard, L. (1983) Reproductive behaviour and development of the dung beetle Typhaeus Typhoeus (Coleoptera, Geotrupidae). *Tijdschrift voor entomologie*, **126**, 203–231.

Edwards, P.B. & Aschenborn, H.H. (1988) Male Reproductive Behaviour of the African Ball-Rolling Dung Beetle, *Kheper Nigroaeneus* (Coleoptera: Scarabaeidae). *The Coleopterists Bulletin*, **42**, pp. 17–27.

Finn, J. a. & Gittings, T. (2003) A review of competition in north temperate dung beetle communities. *Ecological Entomology*, **28**, 1–13.

Finn, J.A., Gittings, T. & Giller, P.S. (1998) Aphodius dung beetle assemblage stability at different spatial and temporal scales. *Applied Soil Ecology*, **10**, 27–36.

Floate, K.D. (2007) Endectocide residues affect insect attraction to dung from treated cattle: implications for toxicity tests. *Medical and veterinary entomology*, **21**, 312–22.

Gittings, T. & Giller, P.S. (1997) Life history traits and resource utilisation in an assemblage of north temperate Aphodius dung beetles (Coleoptera: Scarabaeidae). *Ecography*, **20**, 55–66.

Hanski, I. & Cambefort, Y. (1991) *Dung Beetle Ecology.* Princeton University Press.

Hirschberger, P. (2001) Stridulation in Aphodius Dung Beetles: Behavioral Context and Intraspecific Variability of Song Patterns in *Aphodius ater* (Scarabaeidae). *Journal of Insect Behavior*, **14**, 69–88.

Holter, P. (1979) Abundance and reproductive strategy of the dung beetle *Aphodius rufipes* (L.) (Scarabaeidae). *Ecological Entomology*, **4**, 317–326.

Jessop, L. (1986) *Dung Beetles and Chafers, Coleoptera: Scarabaeoidea. Royal Entomological Society of London, London, U.K.*

Kasper, J. & Hirschberger, P. (2005) Stridulation in Aphodius dung beetles: Songs and morphology of stridulatory organs in North American Aphodius species (Scarabaeidae). *Journal of Natural History*, **39**, 91–99.

Landin, B.-O. (1961) *Ecological Studies on Dung-beetles (Coleoptera: Scarabaeidae).* Berlingska Boktryckeriet, Lund.

Larsson, M.C., Hedin, J., Svensson, G.P., Tolasch, T. & Francke, W. (2003) Characteristic Odor of Osmoderma eremita Identified as a Male-Released Pheromone. *Journal of Chemical Ecology*, **29**, 575–587.

Lobo, J.M. & Martín-Piera, F. (1999) Between-group differences in the Iberian dung beetle species-area relationship (Coleoptera: Scarabaeidae). *Acta Oecologica*, **20**, 587–597.

McDonald, J.H. (2009) *Handbook of biological statistics*. Sparky House Publishing Baltimore, MD.

Menzel, U. (2013) EMT: Exact Multinomial Test: Goodness-of-Fit Test for Discrete Multivariate data.

Njihia, T.N., Jaramillo, J., Murungi, L., Mwenda, D., Orindi, B., Poehling, H.-M., *et al.* (2014) Spiroacetals in the colonization behaviour of the coffee berry borer: a “push-pull” system. *PloS one*, **9**, e111316.

Palestrini, C., Barbero, E. & Ronaldo, A. (1998) Intra- and interspecific aggregation among dung beetles (Coleoptera: Scarabaeoidea) in an Alpine pasture. *Journal of Zoology*, **245**, 101–109.

Penttilä, A., Slade, E.M., Simojoki, A., Riutta, T., Minkkinen, K. & Roslin, T. (2013) Quantifying beetle-mediated effects on gas fluxes from dung pats. *PloS one*, **8**, e71454.

Prokopy, R.J. (1975) Oviposition-deterring Fruit Marking Pheromone in Rhagoletis fausta. *Environmental Entomology*, **4**, 298–300.

R Core Team. (2014) R: A Language and Environment for Statistical Computing.

Roslin, T. (2000) Dung beetle movements at two spatial scales. *Oikos*, **91**, 323–335.

Roslin, T. & Koivunen, A. (2001) Distribution and abundance of dung beetles in fragmented landscapes. *Oecologia*, **127**, 69–77.

Růžička, Z. (2013) Oviposition-deterring pheromone in *Chrysopa oculata* (Neuroptera: Chrysopidae). *European journal of entomology*, **91,** 361-370**.**

Tribe, G.D. (1975) Pheromone release by dung beetles (Coleoptera: Scarabaeidae). *South African journal of science*, **71**, 277–278.

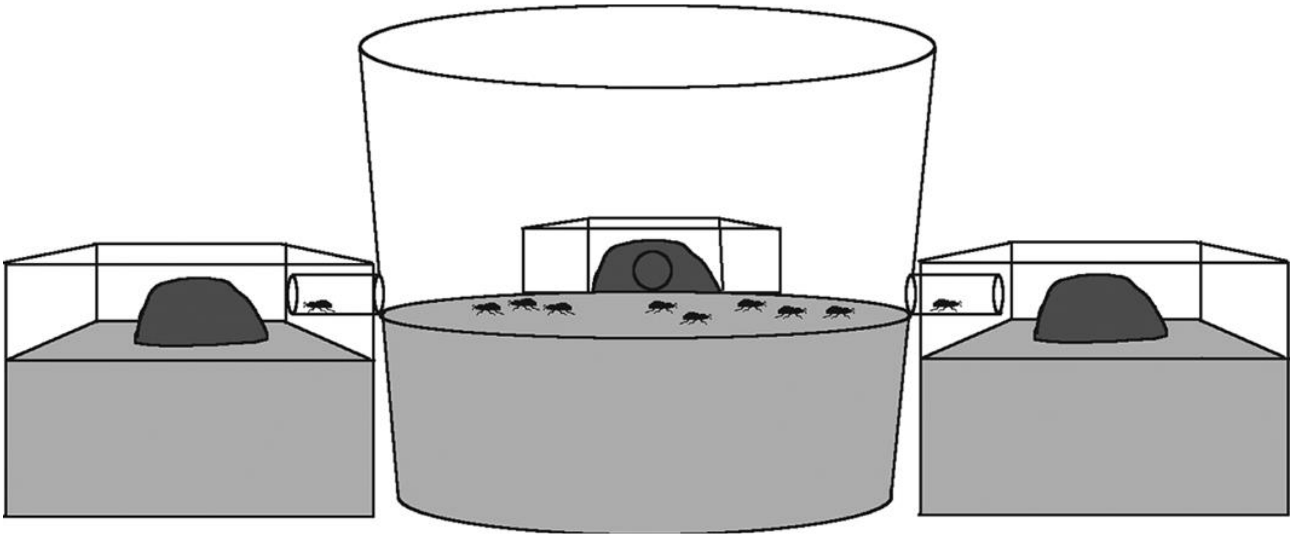

Figure 1. Dung choice experiment apparatus. Opaque tubs and arena are shown as transparent for clarity.

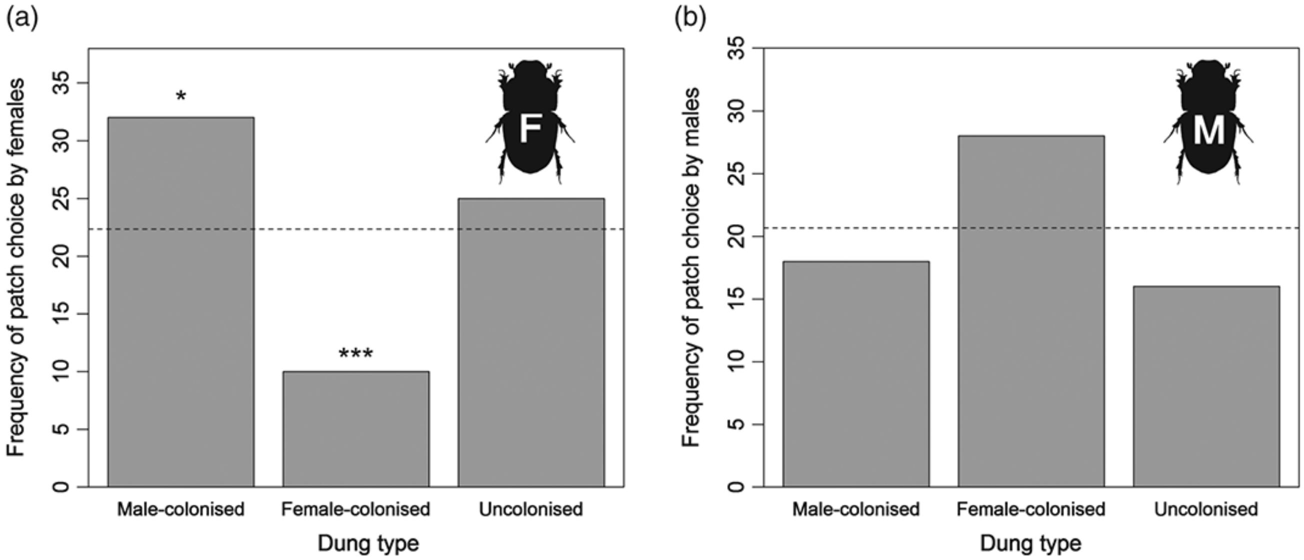

Figure 2. Preference of (a) female and (b) male *Aphodius fossor* in selecting dung which is uncolonised, or colonised by either male or female conspecifics. The dashed line marks the expected number of individual selections based on non-preference. Stars indicate frequencies which are significantly different from expectations of non-preference, values (*P < 0.05, ***P < 0.001).