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Bring out your dead: quantifying corpse removal in *Bombus terrestris*, an annual eusocial insect

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Corpse removal is a hygienic behaviour involved in reducing the spread of parasites and disease. It is found in social insects such as honeybees, wasps, ants and termites, insect societies that experience high populations and dense living conditions that are ideal for the spread of contagion. Previous studies on corpse removal have focused on perennial species that produce thousands of workers, a life history that may incur a greater need for hygienic behaviours. However, whether and how corpse removal occurs in annual species of social insect, which may experience different selection pressures for this behaviour, remains largely unknown. Here the corpse removal behaviour of the bumblebee *Bombus terrestris* was investigated by artificially adding larval and adult corpses into colonies. Larvae were removed more rapidly than adults, with adult corpses eliciting significantly more antennating and biting behaviours. Workers that removed larval corpses were significantly more specialized than the worker population at large, but this was not the case for workers that removed adult corpses. Workers that were previously observed spending more time inactive were slightly, but significantly less likely to perform corpse removal. Size did not affect whether a worker removed corpses, but workers that removed larvae were significantly larger than those that removed adult corpses. Finally, infecting larvae with the virulent parasite *Nosema bombi* did not elicit prophylactic removal. Our results provide the first quantification of corpse removal in an annual social insect and set the scene for comparative analyses of this important behaviour across social insect life histories.

Social insect colonies have evolved arguably the most complex societies in the animal kingdom (Wilson, 1971). Their sophisticated colonies enable ecological dominance (Wilson, 1971), but at the same time this social life comes with a range of costs. One such cost is the disposal of waste, and specifically the disposal of dead colony members (Schmid-Hempel, 1998). As in human societies, where dead individuals are identified by members of the medical profession, removed by undertakers, and buried by grave-diggers or cremated in specialized structures, perennial honeybee, ant and termite societies have been shown to dispose of dead nestmates in a variety of ways. Honeybees remove infected or dead individuals from the hive (Breed, Williams, & Queral, 2002; Trumbo, Huang, & Robinson, 1997; Trumbo & Robinson, 1997; Visscher, 1983), and detect and remove dead or diseased brood (Rothenbuhler, 1964; Spivak & Gilliam, 1993). Similar behaviours have been found in ants, where workers remove dead workers (Arathi, Burns, & Spivak, 2000; Bot, Currie, Hart, & Boomsma, 2001; Choe, Millar, & Rust, 2009; Diez, Borgne, Lejeune, & Detrain, 2013; Diez, Deneubourg, & Detrain, 2012; Diez, Lejeune, & Detrain, 2014; Julian & Cahan, 1999) and pupae (Qiu et al., 2015). Termites either remove (Renucci, Tirard, & Provost, 2011), isolate (Ulyshen & Shelton, 2012) or bury (Chouvenc, Robert, Sémon, & Bordereau, 2012) the dead members of their colony. In both ants and honeybees, such behaviours are often conducted by a set of workers that have been primarily allocated to the task of corpse removal, the so-called 'undertakers' (Julian & Cahan, 1999; Rothenbuhler, 1964). The removal and isolation of dead nestmates is associated with the potential threat of contamination and disease from decaying corpses, which is a particular issue in the densely populated nests of perennial social insects. The evolution of such corpse removal behaviour and task allocation is thus presumably a balance between the costs of not removing corpses and the costs of doing so, modified by the benefits gained from corpse disposal. Previous work has focused on large, complex, perennial societies (see above), which usually have large forces of relatively inactive, reserve workers, and here the benefits of removing corpses clearly outweigh the costs of doing so, or the costs of not disposing of corpses at all.

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The costs and benefits of corpse removal are likely to vary with colony size and longevity. For example, the costs of not removing corpses from the colony are likely to increase with colony longevity, as the longer a colony lives the more such waste will build up. In contrast, the costs of corpse removal, in terms of both the actual energetic removal cost and the allocation of workers to this task, are likely to be relatively lower in large colonies, where large groups of reserve workers exist. Given this, understanding whether corpse removal occurs in small annual eusocial colonies, and, if so, how it is done, may provide insight into the costs and benefits of such behaviour.

Bumblebees provide an ideal model system to address such questions. Colonies have an annual life cycle, existing for a few months from foundation to senescence, and generally consist of tens to a few hundreds of workers. While corpse removal has been observed (Sladen, 1912) or assumed (Jandt & Dornhaus, 2014) in previous studies, such behaviour has not been systematically studied or quantified. Here we used controlled laboratory experiments to address the following questions. (1) Do bumblebees exhibit consistent corpse removal? (2) Does the type of corpse influence removal behaviour? (3) Can we predict, based on size or behavioural profile, which individuals are responsible for corpse removal? (4) Can bumblebees perform prophylactic removal of diseased brood?

METHODS

Study Colonies

Three colonies of *Bombus terrestris audax* were ordered from BioBest (Belgium). These colonies were labelled A, B and C. They were transferred into new plastic observation boxes (29.5 × 23 cm and 14 cm high) and 40 workers were randomly selected, removed and allocated an individual number tag that was glued to their thorax; all other workers were removed. Different coloured tags were used for each of the colonies. During worker removal, while the brood was unoccupied, a larval clump was removed, and 10 approximately equal-sized larvae were extracted from each colony (Colony A: mean length ± SD = 3.8 ± 0.34 mm, $N = 10$; Colony B: 4.8 ± 0.85 mm, $N = 10$; Colony C: 5.5 ± 0.47 mm, $N = 10$). The larvae were stored in individually labelled Eppendorf tubes corresponding to that colony and frozen prior to experiments. The remaining adults that were not allocated a number tag were freeze-killed and 10 of approximately equal size (Colony A: mean thorax width ± SD = 11.4 ± 0.51 mm, $N = 10$; Colony B: 11.4 ± 0.51 mm, $N = 10$; Colony C: 11.9 ± 0.31 mm, $N = 10$) were removed from each colony and stored ready for experiments. After the brood and queens were transferred into the observation boxes, the newly tagged adults were reintroduced to their original colony. The observation boxes were attached to their own individual foraging arena (104 × 79 cm and 52 cm high) with a plastic tunnel (22 × 3.5 cm and 3.5 cm high). These arenas were supplied ad libitum with nectar in plastic dispensers and false flowers made from cardboard and pipe-cleaners that replicated the anther of a flower to which ground pollen was applied by hand. The colonies were given several days to enable a regular foraging pattern to be established; this was identified by foragers venturing into the foraging arena, drinking nectar or collecting pollen and returning straight back to the colony box. Nectar was provided in dispensers that were connected to colony boxes overnight and pollen was added to the nest to ensure larvae were fed if pollen was not foraged from the arenas. Throughout the course of observations newly emerged bees were tagged with a new number tag with the colour corresponding to that colony. A maximum of about 60 bees were tagged from each colony; after this limit was reached untagged bees were then removed and frozen to enable accurate in-colony observations.

Behavioural Observations

Creation of individual level behavioural profile

Each colony was observed for approximately 30 min every morning over 2 weeks. The behaviour of each worker was recorded and allocated to a behaviourally defined 'task' (Table 1). These data were inputted by date and time to create a unique behavioural profile for each individual bee.

Corpse removal trials

The time taken for larvae or adult corpses to defrost was kept constant across experimental trials, as the odour profile of the corpse may change with defrosting time (Diez, Moquet, & Detrain, 2013). For each trial, once the corpse was defrosted it was returned to its original colony onto an area of brood where no bees were within 2 cm. Once the corpse was added a timer was set. Focal animal sampling was used to identify the behaviour displayed towards each corpse by the interacting worker or workers (see Table 1). The tag numbers of the workers that performed the interactions and the times at which these interactions occurred were recorded. Observations stopped when this behaviour resulted in the corpse being deposited in (1) a refuse area within the nest or (2) the foraging arena, if no further interactions were made for 2 min, or if the corpse was lost from view. Individual larval corpses were added into one colony at a time and observed. Adult corpses were then added into each colony and observed. This process was repeated until behavioural observations had been completed for 10 larval corpses and 10 adult corpses per colony. We conducted experimental replicates over a series of successive days, separating repeats of corpse type in individual colonies by approximately 24 h, making short-term reinforcement or specialization unlikely.

Experiments to Test Prophylactic Removal

Nosema bombi is a virulent pathogen of bumblebees (Otti & Schmid-Hempel, 2007, 2008; Rutrecht & Brown, 2009) that is most infective to larvae (Rutrecht, Klee, & Brown, 2007). After eclosion, infected individuals (a proportion of which have crippled wings and thus never leave the nest; Rutrecht & Brown, 2009) shed spores within the nest, leading to an increase in the prevalence, and presumably impact, of the parasite over the colony life cycle (Rutrecht & Brown, 2008). Removal of such infected larvae could be used to control the parasite, and thus this provides an excellent system in which to test for prophylactic brood removal.

Preparation of inoculum

The inoculum was prepared by dissecting the abdomens and extracting the guts of four *B. terrestris* males that had been infected with *N. bombi*. The gut contents from each male bee were placed in an individual Eppendorf tube together with 250 μl of ammonium chloride. This was then crushed using a blunt pipette tip until the solution was mixed and spores of *N. bombi* were suspended. Presence of *N. bombi* was confirmed for each bee by observing 5 μl of each inoculum under a phase contrast microscope at ×400 magnification and scanning for spores. Tubes containing spores were then stored on ice to prevent spores from germinating. To prepare purified inocula, tubes were spun in a balanced cold centrifuge at 4 °C, 5000 rpm for 10 min. Supernatant was removed from each of the tubes using a pipette, taking care not to dislodge the pellet that had formed, and checked for spores. No spores were found so the effluent was discarded. Then, 250 μl of ammonium chloride was added to each of the Eppendorf tubes, which were