The Modified Pharaoh Approach: Stingless bees mummify beetle parasites alive

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Social insect colonies usually live in nests, which are often invaded by parasitic species[1]. Workers from these colonies use different defence strategies to combat invaders[1]. Nevertheless, some parasitic species are able to bypass primary colony defences due to their morphology and behaviour[1-3]. In particular, some beetle nest invaders cannot be killed or removed by workers of social bees[2-5], thus creating the need for alternative social defence strategies to ensure colony survival. Here we show, using Diagnostic Radioentomology[6], that stingless bee workers *Trigona carbonaria*, immediately mummify invading destructive nest parasites *Aethina tumida* alive, with a mixture of resin, wax and mud, thereby preventing

severe damage to the colony. In sharp contrast to the responses of honeybee[7] and bumblebee colonies[8], the rapid live mummification strategy of *T. carbonaria* effectively prevents beetle parasite advancements and removes their ability to reproduce. The convergent evolution of live mummification by stingless bees and social encapsulation by honeybees[3] suggests that colonies of social bees generally rely on, secondary defence mechanisms when harmful nest intruders cannot be killed or ejected easily. This process is analogous to immune responses in animals.

Social insects live in colonies and usually construct nests which are often attractive to parasites. Some parasites feed on stored food or brood and can destroy colonies[3] thus generating the need for efficient defence mechanisms. While some Coleopteran nest intruders are harmless[8-12], others can be damaging parasites[4]. Parasitising beetle species pose particular difficulties for their social insect hosts because their hard exoskeletons protect them from direct primary defence strategies such as biting or stinging. The small hive beetle, *Aethina tumida* (Coleoptera: Nitidulidae), is a parasite and scavenger of honeybee (*Apis mellifera*) colonies endemic to sub-Saharan Africa[2,5,7,13]. It has become an invasive species[14] with well established populations in North America and Australia[13,15]. It lives within *A. mellifera* nests and feeds on brood, stored food and dead bees[5,7,16,17]. Frequently, the feeding small hive beetle larvae cause the complete destruction of the nest[5,7] however, the presence of adult small hive beetles alone can be detrimental to colonies of European honeybees[18]. This obviously creates demand for efficient defence mechanisms against intrusion and reproduction by adult small hive beetles.

Unlike other parasites, small hive beetles are easily detected and can be vigorously attacked by honeybee workers[19]. Nevertheless, adult small hive beetles can bypass primary defences of the bees and easily intrude weak or strong host colonies[5,7] because it is difficult for honeybees to kill or eject them[3,5] due to the beetles' hard exoskeletons and defensive behaviours, such as the turtle defence posture or by dropping from combs[3,7]. Cape honeybees, *A. m. capensis*, display secondary defence mechanisms by encapsulating small hive beetles in tombs made from tree resin (propolis), which the bees collect for use as a nest cavity sealant[3]. Despite the lack of co-evolution between host and parasite, European honeybees also encapsulate small hive beetles in propolis tombs[20] suggesting that encapsulation appears to be part of the general secondary defence of honeybee colonies.

Recent evidence suggests that small hive beetles also parasitise colonies of other social bees. In fact, small hive beetles have been found naturally infesting commercial bumblebee colonies, *Bombus impatiens*, in the field[21] and in greenhouses[8] in North America. Natural small hive beetle infestations were reported in colonies of stingless bees, *Dactylurina staudingerii*, in West Africa[22] and small hive beetle larvae were also observed in a *T. carbonaria* colony that had recently died (Anne Dollin, personal observations) in Australia. Odour cues from stored nest products could attract host-searching adult small hive beetles. We therefore expect colonies of stingless bees to be attractive to small hive beetles and, possibly, suitable for their reproduction. Analogous to honeybees, stingless bees use batumen (a mixture of wax, plant resins and mud) to seal nest cavities[23], thus similar to honeybees, stingless bees may also show alternative secondary defence mechanisms against harmful nest intruders. Here, we evaluated the defence behaviour of an Australian species of stingless bee, *T. carbonaria*, against hive-intruding small hive beetles.

Laboratory reared[24] adult small hive beetles, with $BaSO_4$-marked elytra, were introduced to the entrances of five *T. carbonaria* hives (N=10 each hive) via a transparent plastic tube[3,8]. All hives were CT scanned in a human body scanner (General Electric HiSpeed 64 Slice, General Electric Company) at 5 min intervals for 90 min[25]. To assess small hive beetle distribution within the hives, we used BeeView 3D rendering software (Disect Systems Ltd; Suffolk, UK). Two dimensional images were performed to enable precise measurement of small hive beetle positions and 3D images were performed to provide spatial representation of small hive beetles with respect to hive structures. One hive was randomly selected after scanning and snap frozen with LN_2 for visual screening to compare positions of small hive beetles with respect to scanned images.

Upon introduction of small hive beetles, bees from all *T. carbonaria* hives immediately coated beetles with batumen. The vigorous attacks by workers (Fig. 1) caused the beetles to remain motionless, with their heads tucked underneath the pronotum and legs and antennae pressed tightly to the body (= turtle defence posture[3]). When not attacked, beetles progressed further into the hive. However, most *T. carbonaria* bees continuously attacked the small hive beetles, thereby keeping them in the turtle defence posture. While six small hive beetles did not manage to progress into the hives and were mummified on the spot, others were able to progress further. In one hive, two small hive beetles reached a distance of 170 mm from the hive entrance, just beneath the brood (Fig. 2A). All forward advancements by beetles ceased within 10 min of their introduction into the hive (Fig. 2B). The dissection of one hive confirmed the positions of small hive beetles (N = 10) in relation to its scanned images.

When colonies of social bees are invaded by nest parasites which are difficult to kill or eject, the host colony faces a dilemma. Successful parasite reproduction must be prevented but direct physical attacks alone are not always sufficient to kill defensive opponents like adult small hive beetles[3]. The encapsulation process of adult small hive beetles in honeybee colonies combines prison construction and guarding which usually lasts 1-4 days[3]. Beetles mimic worker bee begging behaviour and are fed by worker bees[27], thus allowing enough time for beetle mating to occur[27]. Our data clearly show that the stingless bees, *T. carbonaria*, use live mummification of parasitic small hive beetles, the “Alternative Pharaoh Approach”, as an effective and fast secondary defence mechanism to prevent successful parasite reproduction. While social encapsulation of small intruders in wax or propolis confinements has been described from *Bombus* and *Apis*[28], to our knowledge, this is the first report of live mummification of nest intruders in colonies of social bees. Our experiment shows that live beetle mummification by *T. carbonaria* takes as little as 10 min Fig. 2B, suggesting that this behaviour can be more effective than that of honeybees. When small hive beetles adopt the turtle defence posture most of the honeybee guards leave the beetles, which then scurry into hiding[3,19]. In contrast, most *T. carbonaria* bees continuously attack the small hive beetles, thereby keeping them in the turtle defence posture. This enables other workers to mummify the beetles alive with batumen whilst they remain motionless Fig.3. Therefore, it appears that the combination of continuous attacks and quick recruitment of mummifying bees underlies this efficient secondary colony defence mechanism of *T. carbonaria*. There have however, been reports of heat-stressed *T. carbonaria* colonies being destroyed by small hive beetles in Australia (Mark Greco, personal observations), suggesting that this invasive species may still pose some threat to native pollinators Fig. 4.

In conclusion, single bees, are not able to kill or eject beetle parasites alone. Only a team with individuals performing specific tasks (e.g. wrestling or gluing in the case of live mummification) can overcome parasite advancements. Live mummification of small hive beetles by stingless bees has probably evolved as a secondary defence mechanism to prevent successful reproduction of nest parasites. This process is a social analogue to immune responses within organisms. It is clearly effective, because small hive beetles are quickly immobilised and prevented from successful reproduction. This seems especially important in light of the high reproductive potential of small hive beetles[24]. The convergent evolution of live mummification of nest parasites in stingless bees and social encapsulation in honeybees is another striking example of evolution between insect societies and their parasites.

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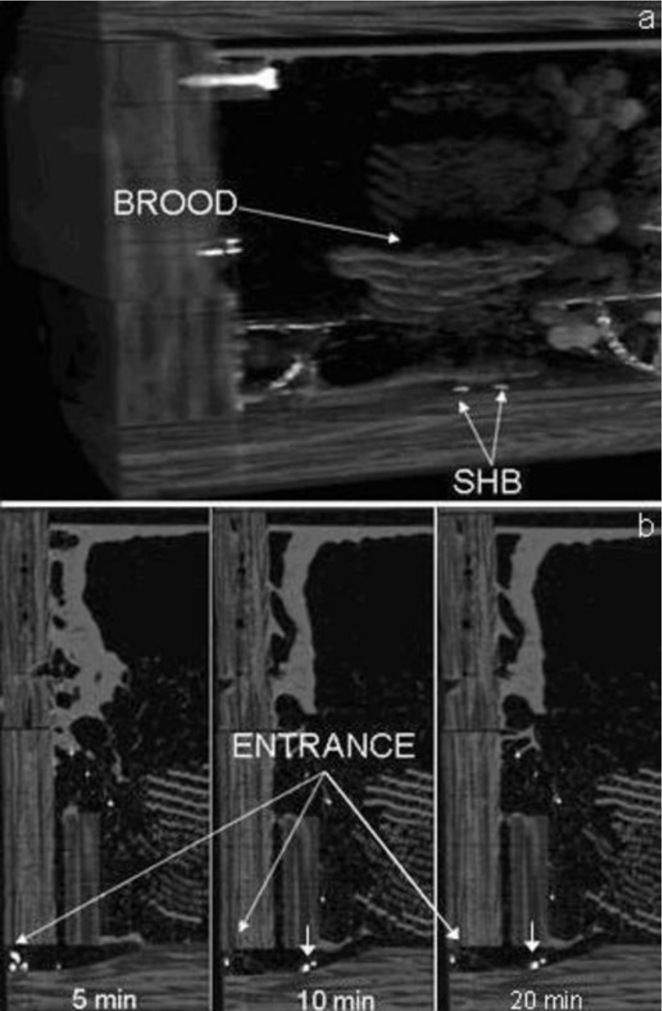
Figure 1: A *T. carbonaria* worker mummifies a live small hive beetle by gluing bits of batumen on its elytra and legs.

Figure 2: Live mummification of adult small hive beetles in *T. carbonaria* hives visualised by CT scans: (a) 3D CT image of *T. carbonaria* brood (single arrow) and two small hive beetles below brood (double arrows); (b) 2D CT image of small hive beetles (short arrows) in entrance of *T. carbonaria* hive demonstrating no change in position after 10 min.

Figure 3: A 3D pseudocolour CT scan image of a *T. carbonaria* hive, detailing brood (b) and live mummified small hive beetles (four white oval bodies) in entrance (e).

Figure 4: Photograph of a *T. carbonaria* hive invaded by reproducing small hive beetles, detailing brood (b) and small hive beetle larvae (L). The hive became vulnerable to invasion after being weakened as a result of extreme ambient temperature (48°C).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