1. INTRODUCTION

Many organisms harbour symbiotic associates in their gut, body cavity, tissues or cells. Some obligate symbionts are mutualists that contribute to the fitness of their host, whereas other facultative symbionts are commensals or parasites that tend to cause negative effects on their hosts (cf. Bourtzis & Miller 2003).

In parasitic associations, the evolutionary interest of the symbiont contradicts that of the host, which sometimes causes the phenomena so-called 'parasite manipulation' wherein symbiont infection alters host behaviour to facilitate its vertical/horizontal transmission. In mutualistic associations, meanwhile, symbiont-induced alteration of host behaviour that enhances its transmission has been little described. Here we report such a case in the stinkbug *Megacopta punctatissima* associated with an obligate gut bacterium. When female stinkbugs lay eggs, small particles called 'symbiont capsules' are deposited underside of the egg mass. Newborn nymphs immediately acquire the symbiont from the capsule, and then aggregate and become quiescent. By manipulating the levels of symbiont supply to newborn nymphs experimentally, we demonstrated that (i) experimental depletion of the symbiont resulted in the occurrence of wandering nymphs, (ii) the less symbiont supply, the more wandering nymphs, and (iii) almost all wandering nymphs were either symbiont-free or symbiont-depleted, whereas the majority of resting nymphs were infected with sufficient titres of the symbiont. These results strongly suggest that the nymphal behaviour is strongly influenced by the success/failure of the symbiont acquisition, thereby ensuring transmission of the essential symbiont and minimizing the energy and time spent for the activity.

**Keywords:** *Megacopta punctatissima*; Ishikawaella capsulata; symbiont capsule; vertical transmission; behavioural manipulation

2. MATERIAL AND METHODS

(a) Field collection of egg masses

Freshly deposited egg masses of *M. punctatissima* were collected from the kudzu vine, *Pueraria lobata*, at Tsukuba, Japan from May to June in 2003 and 2005, by inspecting buds of wild plants every day.

(b) Experimental manipulation of egg masses

From each of the field-collected egg masses, eggs and capsules were carefully removed by using fine forceps under a binocular microscope, whereby experimental egg masses with 5–15 eggs and a capsule were generated.

(c) Observation of nymphal behaviour

Each of the manipulated egg masses was glued onto a piece of filter paper, placed in a plastic Petri dish humidified with a wet cotton ball and incubated at room temperature. Under this condition, eggs in an egg mass synchronously hatched within several hours on the seventh day after oviposition (T. Hosokawa 2003–2005, personal observation). We recorded the nymphal behaviour within 12 h after hatching, the nymphs immediately probe the capsules to acquire the symbiont, get into a resting status for 1–2 days in aggregation (figure 1a) and then disperse to feed on plant sap (Hosokawa et al. 2005, 2006, 2007). Here we report that in *M. punctatissima*, sufficient symbiont acquisition induces nymphal resting behaviour in aggregation while insufficient symbiont acquisition results in nymphal wandering behaviour.

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3. RESULTS

(a) Aggregating behaviour of newborn nymphs in normal egg masses without manipulation

Newborn nymphs of *M. punctatissima* from field-collected egg masses immediately probed symbiont capsules with their proboscis for approximately 1 h, and then aggregated and became quiescent near the eggshells (figure 1a). Diagnostic PCR detection confirmed that all the nymphs successfully acquired the symbiont from the capsules (data not shown).

(b) Experimental depletion of the symbiont resulted in the occurrence of wandering nymphs

By removing eggs and capsules from field-collected egg masses, we generated 260 experimental egg masses with 5–15 eggs and a single capsule, whereby the levels of symbiont supply per nymph were controlled. Note that egg masses with five or six eggs meet the minimal requirement for the symbiont, while egg masses with seven or more eggs suffer depleted symbiont supply at different levels (cf. Hosokawa *et al.* in press). In these experimental egg masses, many nymphs failed to exhibit normal resting behaviour, either actively wandering in the rearing container or getting immobile singly or in a group of a few insects (figure 1b).

(c) The less symbiont supply, the more wandering nymphs

Figure 1c shows the relationship between the level of symbiont depletion and the number of wandering nymphs. In the experimental egg masses with five nymphs per capsule, few nymphs exhibited wandering behaviour. However, as the number of nymphs per capsule increased, the more wandering nymphs occurred.

(d) The majority of wandering nymphs were symbiont-free

Then, we generated 24 experimental egg masses consisting of 15 eggs and a capsule, and investigated the relationship between the infection status of newborn nymphs and their behaviour. Of the 360 newborn nymphs in total, 295 were symbiont-positive whereas 65 were symbiont-negative. The wandering nymphs occupied nearly 80% of the symbiont-negative insects and accounted for only 20% of the symbiont-positive insects (figure 2a).

(e) Almost all wandering nymphs were symbiont-depleted

Figure 2b,c shows the distribution of symbiont titres detected in the resting and the wandering nymphs, respectively. A previous study demonstrated that nymphs of *M. punctatissima* normally acquire $2 \times 10^7$ symbionts on average, and the minimal symbiont titre needed for normal development of the nymphs is approximately $2 \times 10^6$ (Hosokawa *et al.* in press). Quantitative PCR assays revealed that most of the resting nymphs (93.3%; 235 out of 252) were infected with the symbiont, the majority of the infected nymphs exhibited symbiont titres over the threshold level of $10^6$ and the distribution peak was at the normal acquisition titre, approximately $10^7$ (figure 2b). By contrast, only 55.6% of the wandering nymphs (60 out of 108) were infected with the symbiont and most of the infected nymphs exhibited symbiont titres below $10^6$ (figure 2c).

4. DISCUSSION

These results indicate that in *M. punctatissima*, sufficient symbiont acquisition induces nymphal...
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resting behaviour in aggregation while insufficient symbiont acquisition results in nymphal wandering behaviour. It is expected that the behavioural pattern of the nymphs ensures transmission of the essential symbiont and minimizes the energy and time spent for the activity.

In the case of M. punctatissima, it may be simpler and parsimonious to assume that the behavioural alteration associated with the symbiont acquisition is an adaptive behavioural response of the host insect rather than a consequence of behavioural manipulation by the bacterial symbiont. However, considering that the host and the symbiont are both benefited from the behaviour, the behaviour might have been evolutionarily favoured by selection pressures acting on both the partners.

What factor is responsible for the behavioural alteration is currently unknown. In the symbiont capsule of M. punctatissima, symbiont cells and a secretion matrix are encased (Hosokawa et al. 2005). Probably the nymphs are sensitive to some chemical or cellular component of the symbiont or the secretion matrix. Alternatively, the nymphs may simply perceive the quantity of what they ingested or the time of their probing. Some of the wandering nymphs were not free of the symbiont but their infection titres were below the threshold level (figure 2c), suggesting that the nymphs can somehow monitor the quantity of what they ingest from the symbiont capsule.

Young nymphs of stinkbugs generally exhibit gregariousness. In the stinkbug Nezara viridula, the gregariousness was reported to improve developmental performance of the nymphs (Kiritsani 1964; Lockwood & Story 1986). It has been argued that the nymphal gregariousness might enhance aposematic effects against predators (Aldrich & Blum 1978). However, actual biological significance of the resting/aggregating behaviour in stinkbug nymphs has been obscure. Although speculative, the resting behaviour might be involved in the initial establishment of the symbiosis in the midgut.

In diverse insect–microbe mutualisms, the host insects have developed elaborate molecular, cellular, morphological and/or developmental traits for ensuring association with their indispensable partners, such as formation of specialized cells (e.g. bacteriocytes) and organs (e.g. bacteriomes, midgut crypts, mycangia) for harbouring the symbionts (Buchner 1965), specific gene expression in these cells and organs (Nakabachi et al. 2005), sophisticated mechanisms for vertical transmission of the symbionts (Miura et al. 2003; Hosokawa et al. 2005), etc. This study highlights the possibility that specialized behavioural traits also contribute to the maintenance of intimate host–symbiont associations, and thus can evolve for that purpose. We expect that, in addition to the well-known cases of parasite-induced behavioural alteration (Moore 2002; Thomas et al. 2005), symbiont-mediated alteration of host behaviour might be more common among mutualistic associations than previously envisioned, particularly wherein symbiont transmission entails behavioural elements.


