Biological characterization of the obligate symbiosis between *Acropyga sauteri* Forel (Hymenoptera: Formicidae) and *Eumyrmococcus smithii* Silvestri (Hemiptera: Pseudococcidae: Rhizoecinae) on Okinawa Island, southern Japan

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Abstract
The ant *Acropyga sauteri* Forel has an obligate, mutualistic symbiosis with a mealybug, *Eumyrmococcus smithii* Silvestri, on Okinawa Island, southern Japan. The mealybugs live inside ant nests nearly all their lives, and the ants depend on them for food. Alate foundress queens carry mealybugs during their nuptial flights, using them to establish new colonies at new sites. However, important aspects of the symbiosis have not yet been elucidated. The present study characterizes the basic biology of the symbiosis and describes for the first time the morphologies of all growth stages of *E. smithii*. Our study suggests that *E. smithii* has only one nymphal stage, followed by a female pupal stage or male prepupal stage. Intensive sampling of ant nests across seasons showed that *A. sauteri* prefers nest sites 5–20 cm underground. *Acropyga sauteri* produced reproductive stages mainly in mid-March or early April, and numbers of both ant workers and mealybugs increased from spring to summer. Experimental determination of colony identity with a method using nestmate recognition by ants suggested that each ant colony rarely has a perimeter greater than 30 cm, that the ants are monogynous, and that different ant colonies are densely aggregated along the root system of a plant, adjacent to each other but not interflowing. Both symbiotic partners were vulnerable to attacks by several common subaerial ant species following physical disturbance to their nests.

Keywords: Ant–hemipteran interactions, life cycle, morphology, mutualism, myrmecophile, myrmecophilous mealybugs, Ryukyu

Introduction
Mutualistic interactions between ant and hemipteran species are not uncommon (e.g. Way 1963; Buckley 1987; Hölldobler and Wilson 1990; Gullan and Kosztarab 1997). Hemipterans provide honeydew for ants in exchange for protection against parasites and...
predators. These interactions are very diverse with regard to the richness of species involved, the degrees of specificity, and the patterns of mutualistic relationships (Way 1963; Buckley 1987; Gullan 1997; Itioka and Inoue 1999; Sakata 1999). Some interactions are very loosely facultative: ants may casually prey on their partners under certain circumstances (Way 1954, 1963; Pontin 1958; Sakata 1995). Some ants harbour their partner hemipterans in their nest only temporarily (e.g. Way 1963; Hölldobler and Wilson 1990), whereas some interactions are obligate: the ants and their partners live together throughout all or most of their life cycles (e.g. Maschwitz and Hänel 1985; Hölldobler and Wilson 1990; Johnson et al. 2001).

It is known that Acropyga Roger ants have mutualistic obligate symbioses with root mealybugs of the genera Eumyrmococcus Silvestri, Xenococcus Silvestri, Neochavesia Williams and Granara de Willink, and others within the subfamily Rhizoecinae of the Pseudococcidae (Silvestri 1927; Weber 1944; Beardsley 1970; Williams 1970, 1978, 1988, 1993, 1998, 2004; Williams and Terayama 2000; Johnson et al. 2001). Acropyga–mealybug symbioses have been found in temperate and tropical areas (Silvestri 1927; Takahashi 1934; Weber 1944; Beardsley 1970; Williams 1970, 1978, 1988, 1993, 1998, 2004; De Lotto 1977; Buschinger et al. 1987; Terayama 1988; Taylor 1992; Williams and Terayama 2000; Johnson et al. 2001). Acropyga ants are subterranean and rarely observed above ground except during nuptial flights, when the reproductive stages appear. An alate foundress queen of Acropyga carries an individual mealybug in its mandibles during the nuptial flight, transferring it to the new nest site (Uye 1928, 1933; Takahashi 1934; Weber 1944; Eberhard 1978; Buschinger et al. 1987; Terayama 1988; Johnson et al. 2001). The life-history traits of the species involved in this association have not been well elucidated—only a small portion of life history following nest founding has been reported from a few systems in South America (Bünzli 1935; Weber 1944; Flanders 1957).

The mealybug genus Eumyrmococcus, which contains 18 described species, is distributed in southern and eastern Asia, Australasia, Europe, and South Africa (Williams 1998; Williams and Terayama 2000). Not all growth stages have been completely described for the 18 species (Williams 1998). In most male Pseudococcidae, two nymphal stages are followed by prepupal, pupal, and adult stages (Williams 1998). In most female Pseudococcidae, three nymphal stages are followed by an adult stage (Williams 1998). In Eumyrmococcus, the third-instar female is a pupa (Williams 1998). The female pupal stage is an “unusual instar” (Williams 1998), and this feature is shared by Xenococcus and Neochavesia, also associated with Acropyga ants (Williams 1988, 1998, 2004). The complete life cycles of these Acropyga symbiont mealybugs, however, have not been revealed.

Symbiotic interactions between an ant species, A. sauteri Forel, and a mealybug species, E. smithii Silvestri, are found on Okinawa Island, southern Japan (Terayama 1988). Except for accidental cases, this mealybug species is found only in A. sauteri nests, where it feeds on plant roots (Uye 1933; Takahashi 1934; Williams 1970; Terayama 1988). The alate founndress queen of A. sauteri carries an individual of E. smithii during its nuptial flight, transferring it to a new nest site (Uye 1928, 1933; Terayama 1988). Only the adult female morphology of E. smithii has been described (Silvestri 1927; Williams 1970, 1978, 1998), as the subterranean nature of the relationship has prevented further characterization of the symbiosis. Clarification of important biological aspects is necessary for a more comprehensive understanding of the evolution of obligate symbioses, such as between Acropyga ants and their symbiont mealybugs.

This study sought to characterize the biology of the symbiosis between A. sauteri and E. smithii. In particular, we attempted to describe the morphologies of the growth stages and
life cycle of *E. smithii*, the spatial distribution and structure of nests of *A. sauteri*, the within-nest distribution of both partners, and the seasonal changes in age structure for both partners. In addition, we investigated other ant species occurring around the nests of *A. sauteri* and their effects on the symbiosis.

**Materials and methods**

**Study site**

The study site (26°10′N, 127°45′E, 65 m in altitude) was in Sashiki Town, the southern part of Okinawa Island, which is a grassland dominated by a perennial grass species, *Miscanthus sinensis* Anderss, but has a mixture of several grass and shrub species. The study site was on a hillside surrounded by secondary forests and sugarcane fields. A plot (25 × 3 m) was established among dense growth of *M. sinensis* (average plant height: ca 2.0 m).

**Ants and mealybugs**

The body length of an *A. sauteri* worker is 2.0–2.5 mm (Yamane et al. 1999). The body of the adult female of *E. smithii* is white in colour, 1.7 mm in length (Silvestri 1927), and has a dilated cephalothorax and an abruptly narrowing abdomen with a dorsally curled tip (Silvestri 1927; Williams 1970, 1978, 1998). The mealybugs were observed feeding on the roots of a species of bamboo (*Uye 1933*), *Saccharum officinarum* L. (Takahashi 1934; Williams 1970; Terayama 1988) and *Imperata* sp. (Takahashi 1934). At the study site, the mealybugs commonly feed on the roots of *M. sinensis*.

Both *A. sauteri* and *E. smithii* are distributed in Japan, Taiwan, and southern China (Silvestri 1927; Takahashi 1934; Williams 1970, 1978, 1998; Terayama 1988).

**Examination of the growth stages of Eumyrmococcus smithii**

To observe the morphology of all growth stages of *E. smithii*, we collected 262 individuals by sampling cubic clods from the ground surface to a depth of 0–15 cm in randomly chosen quadrats in the study plot in April 2000. All collected individuals were prepared on microscope slides by the method of Takagi (1970) with Kawai’s (1980) improvement. For each individual, we measured the width and length of the body and antennae, the width of the spiracle, the length of the hind legs (trochanter, femur, tibia, tarsus, and claw) and body setae, and the width and length of the labium (if present). In individuals with anal rings, we also measured the width of the base of the abdominal segment, the width of the anal ring, and the lengths of anal lobe setae and anal ring setae.

Examination with magnification showed that some of the specimens had the exoskeletons of subsequent stages within their bodies, which indicated that they were just about to moult. Based on the morphology of the mealybugs measured in this study and the morphology of the conspecifics and congenerics described by Williams (1970, 1978, 1998), we attempted to identify the growth stages of these mealybugs.

**Spatial distribution of both partners**

To clarify the spatial distribution of nests of *A. sauteri* and the within-nest distribution of *A. sauteri* and *E. smithii*, we mapped ant nests in the field by digging out clods from the
ground (ranging from 0 to 30 cm in depth) and checking for the presence of both partners in the nest in each clod. We removed 184 cubic clods (5 × 5 × 5 cm) in a randomly selected 55 × 25 cm area (ranging from 0 to 20 cm in depth) in August 2001, and 212 cubic clods (5 × 5 × 5 cm) in three randomly selected 45 × 45 cm areas and a 45 × 75 cm area (ranging from 0 to 30 cm depth) in June 2002 (Figure 1a; Table I). We recorded the locations of the quadrats, and all the cubic clods were mapped on the grid in each quadrat (Figure 1a). Each clod was transported to the laboratory in a plastic bag. All ants and mealybugs in each cubic clod were then collected. Only the August 2001 samples were used for analysis of the spatial distribution of the nests in relation to the depth, but all the samples were used for the other analyses.

Determination of colony identity and spatial range of a single colony

Ant nest tunnels ran along the root systems of host plants, crossing reticulately, and the colonies were very densely distributed. It was consequently extremely difficult to recognize the boundaries of neighbouring colonies of *A. sauteri* by finding an uninhabited area between two ant aggregations situated in neighbouring clods. We used ant nestmate recognition behaviour (shown by ant workers in many ant species) to determine the colony identity of two ant worker aggregations that were haphazardly selected. Workers of most ant species are known to show some antagonistic behaviour against non-nestmate conspecifics (Hölldobler and Wilson 1990). If antagonistic behaviour was observed when a worker of one ant aggregation was experimentally introduced into another aggregation, we considered that the two ant aggregations belonged to different colonies. Conversely, if receptive behaviour was observed, we considered the two aggregations to be parts of the same colony. We tested the validity of this method for *A. sauteri* by examining whether *A. sauteri* workers showed distinct antagonistic behaviour toward a worker experimentally introduced from a different colony and receptive behaviour toward a worker from the same colony. We removed 18 cubic clods (10 × 10 × 10 cm) in 12 randomly located quadrats (10 × 10 cm, ranging from 0 to 20 cm in depth) in January 2002 (Figure 1b; Table I). In the laboratory, we collected eight aggregations from the clods. Each ant aggregation was derived from a single cavity or tunnel. For each aggregation, five to seven workers were placed in a plastic cup (60 cm³) covered with black paper (Figure 2b) and rested until they resumed normal behaviour. Then we arranged 14 inter-aggregational experimental matches (Figure 2c). In each match, we introduced an ant worker from one cup (aggregation) into another cup (aggregation) and observed the behaviour stimulated by the introduced worker (Figure 2d). The interactions between the ants were classified as follows: ants from one aggregation may have been (1) bitten, (2) chased, (3) groomed with mouthparts, or (4) ignored by ants from another aggregation. The behaviour of the former two was regarded as “antagonistic”, whereas the behaviour of the latter two was regarded as “acceptive”. After the workers from different aggregations had contacted each other two or three times, we removed the introduced worker and replaced it in its original cup (Figure 2e). We never used an introduced worker for more than one trial. For a match, these trials of introduction (Figure 2c–e) were repeated at least seven times. For all matches, all trials for a match were consistently categorized as a single type of behaviour, as either antagonistic or acceptive. We therefore concluded that this method was effective for the determination of colony identity between ant aggregations derived from two locations.

Using this method (Figure 2c–e) on the ant samples collected in June 2002 (mentioned above; Table I), we checked the colony identity of neighbouring ant aggregations to
Figure 1. Schematic design of cubic clod sampling. (a) Protocol for sampling in June 2002; (b) protocol for sampling in January 2002. At each sampling event we randomly chose a ground surface area for sampling clods, from which individuals of Acropyga sauteri and its symbiont Eumyrmococcus smithii were collected. The dates of sampling events and the numbers and sizes of areas and cubic clods are listed in Table I.
determine the range of a single colony in the clods. For the experiment of colony identity, we collected 29, 32, and 34 ant aggregations from cubic clods in the three different study areas. They were selected in such a way that the distances \((D)\) between two clods containing the subjective two aggregations varied from 5 to 30 cm within each selected area (Figure 2a). In addition, we set up matches between aggregations from different study areas so that the distances between the two aggregations were much more than 30 cm. A total of 96 experimental matches of two aggregations with the use of 95 ant aggregations were conducted. Trials (Figure 2c–e) were repeated five times for each match between two particular ant aggregations.

**Seasonal changes in age structures for both partners**

We examined the seasonal changes in age structure for *A. sauteri* and *E. smithii* using the samples taken in August 2001 and January and June 2002 (Table I). In addition, we collected extra ants and mealybugs in 12 cubic clods (\(15 \times 15 \times 15\) cm) in 12 randomly positioned quadrats (\(15 \times 15\) cm, ranging from 0 to 15 cm in depth) in October 2001, in three cubic clods (\(15 \times 15 \times 15\) cm) in three randomly positioned quadrats (\(15 \times 15\) cm, ranging from 0 to 15 cm in depth) in March 2002, and in five cubic clods (\(10 \times 10 \times 10\) or \(15 \times 15 \times 15\) cm) in two randomly positioned quadrats (\(30 \times 30, 50 \times 50\) cm, ranging from 0 to 15 cm in depth) in April 2002 (Table I).

Several colonies of *A. sauteri* often aggregate densely in a small space, such as the root system of a host plant (see Results). In this case, it was extremely difficult to specify the spatial range of a single colony of *A. sauteri* by recognizing the intervals between ant colonies. Therefore, when multiple colonies might possibly be sampled from certain clods in the vicinity, we estimated the average numbers of ants and mealybugs at different growth stages for the colonies in the clods by dividing the total numbers of ants and mealybugs at different growth stages by the total number of ant queens present in the clods. The validity of this estimation was based on the assumption that *A. sauteri* forms monogynous colonies, which seemed to be a safe assumption for the following reasons. First, Uye (1933) observed

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### Table I. Protocol for sampling cubic clods in the field in order to determine the seasonal changes of age structures of both partners, the spatial distribution of both partners, and the colony identity of ant aggregations (we used the samples at all sampling events for the seasonal changes, those at two events for the spatial distribution, and those at two events for the colony identity).

| Date           | Size of an area for sampling cubic clods (cm) | No. of areas | Size of a cubic clod (cm) | No. of cubic clods |
|----------------|---------------------------------------------|--------------|--------------------------|-------------------|
| August 2001<sup>a</sup> | 55 × 25                                      | 1            | 5 × 5 × 5                | 184               |
| October 2001   | 15 × 15                                      | 12           | 15 × 15 × 15             | 12                |
| January 2002<sup>b</sup> | 10 × 10                                      | 12           | 10 × 10 × 10             | 18                |
| March 2002<sup>c</sup> | 15 × 15                                      | 3            | 15 × 15 × 15             | 3                 |
| April 2002<sup>c</sup> | 30 × 30                                      | 1            | 15 × 15 × 15, 10 × 10 × 10 | 5                 |
|                 | 50 × 50                                      | 1            | 15 × 15 × 15, 10 × 10 × 10 | 5                 |
| June 2002<sup>a,b</sup> | 45 × 75                                      | 1            | 5 × 5 × 5                | 52                |
|                 | 45 × 45                                      | 2            | 5 × 5 × 5                | 68, 92            |

<sup>a</sup>The samples were used for specifying spatial distribution of both partners.  
<sup>b</sup>The samples were used for determining the colony identity of ant aggregations.  
<sup>c</sup>The samples contained several queens in one cubic clod. Therefore we estimated the average numbers of ants and mealybugs per colony by dividing the total number of queens into the total numbers of ants and mealybugs.
colony foundation by solitary queens in this species, and the number of solitary queens drastically increases just after the putative nuptial flight season (see Results). Second, antagonistic behaviour between workers, which would indicate contact between non-nestmates, were frequently observed when multiple queens were found in a small space (see Results). Moreover, antagonistic behaviour was always observed between workers derived from two distant points (see Results). Third, throughout our study, multiple queens in the same nests and intimate interaction between queens were never observed.

Figure 2. Schematic illustration of the method used to determine colony identity. (a) Distance \( D \) between two particular ant aggregations was defined as the distance between the centre of the two clods containing the two aggregations; (b) five workers were placed in a plastic cup covered with black paper; (c) an ant worker was introduced into another cup (of recipient workers); (d) the contact behaviour of recipient and introduced workers was observed; (e) the trial ended after contact had occurred two or three times, after which the introduced worker was returned to the original cup. In a match between two aggregations, the method described from (c) to (e) was repeated five times.
Ants and mealybugs in clods that were more than 30 cm away from the nearest clod containing a queen were omitted from the analysis because they may not have been a representative fraction of a colony.

We statistically compared the total numbers of individuals of *A. sauteri* and *E. smithii* by one-way analysis of variance. We could not statistically analyse the numbers of alate females and males of *A. sauteri* because the sample size was insufficient and because the data were skewed. Instead, we presented the distribution of variables for each colony (sometimes variables estimated from multiple colonies).

*Other ant species*

We censused ants species other than *A. sauteri* in the study plot during every period of clod sampling. We recorded the nest locations of the other ant species, observed their behaviour with special reference to their responses to *A. sauteri* and *E. smithii*, and collected specimens for species identification. We identified the ants using keys provided by Yamane et al. (1999).

*Results*

*Features of Eumyrmococcus smithii*

Mealybugs were morphologically allocated into one of six growth stages by microscopic examination. One stage (39 individuals) was considered to represent adult females, and some of these mealybugs contained eggs. Adult females could additionally be distinguished from the other five stages on the basis of their anal characteristics (e.g. width of anal ring, anal ring setae, and anal lobe setae). Two individuals were determined to be adult males on the basis of their genitalia and the absence of a labium (Appendix; Figure 11).

Three stages were characterized by pupa-like morphologies, which were similar to those described by Williams (1998) for the prepupae and pupae of congeneric species. One of the stages (19 individuals) was identified as the female pupal stage (Appendix; Figure 12a), because the mealybugs contained the exoskeletons of female adults (Figure 3a). Similar to patterns observed in congeneric species (Williams 1998), female pupae at this stage had a labium and longer antennae than observed in the other two pupa-like stages. Of the two remaining pupa-like stages, the antennae of one group of mealybugs was shorter than those of the other, and three of these mealybugs (of a total of seven individuals) contained male adult exoskeletons (Figure 3b). They were thus identified as male pupae (Appendix; Figure 12c). Two (of a total of 15) individuals in the remaining pupa-like group contained the exoskeletons of male pupae, and this stage was therefore considered to represent the male prepupa (Appendix; Figure 12b).

The remaining growth stage (180 individuals) was identified as nymph. One sampled individual had died during emergence from an egg and was not yet sclerotized. Although details of its morphology were not clear because of the manner of death, strong similarities in spiracle width and width of the anal ring between this individual and the remaining 179 nymphs were observed. Consequently, all 180 nymphal individuals were categorized as first-instar nymphs (Appendix; Figure 13). The variations in body size and morphological characteristics, including setal characteristic, observed within this group were continuous and unimodal. Two of these nymphs contained the exoskeletons of female pupae, and 10 contained exoskeletons of male prepupae, which confirmed that this first instar was
followed by a male prepupal stage or a female pupal stage (Figure 4). The morphology of the first-instar nymphs, female and male pupae, male prepupa and male adult are described in the Appendix.

Spatial distribution of nests of Acropyga sauteri

*Acropyga sauteri* workers were distributed at a depth of 0–38 cm in August 2001; no worker was found at depths greater than 40 cm. The numbers of clods with more than five workers varied significantly among depths ($\chi^2=10.837$, $P<0.05$, chi-square test of independence) and was lowest at soil depths of 0–5 cm (Figure 5). All three queens were collected at 10–20 cm depth. The shortest distance between queen-containing clods was 5 cm.

Figure 3. Pupae containing the exoskeletons of adults. (a) Female; (b) male.
Figure 4. Schematic illustration of the presumed life cycle of *Eumyrnococcus smithii*. The first-instar nymph is followed by the pupa and adult in the female and by the prepupa, pupa, and adult in the male.

Figure 5. Percentage of clods containing more than five workers of *Acropyga sauteri* across depths. Numerals above the bars indicate the sample size (number of cubic clods).
The average number of ant workers in clods with *E. smithii* was significantly higher than the number of ant workers in clods without *E. smithii* (Figure 6; August; \( U = 1110.00, P < 0.0001 \), June; \( U = 1420.00, P < 0.0001 \), Mann–Whitney \( U \) test). No individual of *E. smithii* was found in clods in which *A. sauteri* was absent.

**Colony identity and spatial range of a single colony**

In matches between two ant aggregations, done for the purposes of colony identification, recipient and introduced workers consistently showed similar responses in all trials; all displayed either antagonistic or acceptive behaviour. When the distance between two clods containing ant aggregations ranged from 5 to 30 cm, the frequency of acceptive behaviour was 38–47% (Figure 7). The greatest distance between two clods containing ant aggregations with workers displaying acceptive behaviour was 25 cm. When the distance between two clods was more than 30 cm, antagonistic responses were observed in every trial in all matches (Figure 7). Only one queen was found within this presumed spatial range of a single colony (except in the case of new alate foundress queens).

**Within-nest distribution of both partners**

Ant nests had tunnels 1–2 mm in diameter and two types of chambers, both ca 4–7 mm in width and height. One chamber type was penetrated by small plant roots, on which adults and nymphs of *E. smithii* were often observed to be settled. The other chamber type did not contain any plant roots. In these chambers, *A. sauteri* and *E. smithii* individuals at various growth stages were often huddled in clusters. Clusters consisted mainly of *E. smithii* at pupal or prepupal stages and eggs or brood of *A. sauteri*.
Seasonal changes in age structures for both partners

Alate female ants were found in two of three sampled cubic clods in March 2002, although alate females were not found in five of eight colonies. The average number of alate females in the three colonies in March was rather high compared with the number of alate females observed in some colonies in other months (Figure 8a).

Alate males were found only in August 2001, March 2002, and April 2002. Only one alate male was found in August and April (Figure 8b).

Five single queens, unattended by workers, were found only in April 2002.

Workers and larvae of *A. sauteri* were present in all seasons (Figure 9). Pupae were found in August and October 2001 and in June 2002. The proportion of pupae among individuals in a colony was highest in June, whereas only one pupa was found in October 2001 (Figure 9).

The average number of ants in all stages (alate female, alate male, worker, pupa, and brood) per colony was significantly higher in October 2001 than in January, March, and April 2002 (Figure 9; \(P<0.05\), one-way ANOVA followed by Tukey–Kramer test). Average numbers were lowest in January 2002 (Figure 9).
Figure 8. Distribution of the numbers of alate female ants (a) and alate male ants (b) per colony of *Acropyga sauteri*. A plot represents the variable for a colony or the average for multiple colonies in the vicinity. When multiple queens were sampled from certain clods in the vicinity, we estimated the average numbers of the reproductives by dividing the total number of queens into the total numbers of the reproductives. Numerals above the solid circles indicate the number of colonies used for the average estimation.
The average number of mealybugs at all stages (female adult, male adult, pupa, and nymph) per ant colony tended to increase from spring (March) to summer (August) and then decrease during winter (Figure 10). The number of mealybugs per colony in March 2002 was significantly lower than in August 2001 and in April and June 2002 (Figure 10; P<0.05, one-way ANOVA followed by Tukey–Kramer test).

There were seasonal changes in the female adult: nymph ratio corresponding to changes in the total number of mealybugs. The ratio was relatively low as the total number of mealybugs per colony increased from March to June, whereas it was relatively high when the total number of mealybugs per colony decreased from August to January (Figure 10).

Although abundant adult females and nymphs of *E. smithii* were observed in the nests of *A. sauteri* year-round, the occurrence of adult males of *E. smithii* was relatively limited. The males were observed in August 2001 and June 2002, and only one male was found in March 2002 (Figure 10). Pupae of both sexes and male prepupae were observed in all seasons except March 2002 (when they were not found at all; Figure 10).
Other ant species

At least nine ant species other than *A. sauteri* were found in the soil around nests of *A. sauteri* (Table II). Of the nine species, *Camponotus kaguya* Terayama, *Monomorium sechellense* Emery, *Oligomyrmex hannya* Terayama, *Paratrechina* sp., and *Tetramorium biacarinatum* (Nylander) had nests around those of *A. sauteri* (Table II). At least 10 nests of any of the five species were observed at soil depths of 0–30 cm in August 2001 and June 2002 (Table II). Seven nests were observed at 0–5 cm in depth (Table II).

*Pheidole noda* F. Smith and *T. biacarinatum* were often observed invading nests of *A. sauteri* through holes that were accidentally made during sampling activities. These ants carried individuals of both *A. sauteri* and *E. smithii* out in their mandibles. Except when disturbed nests of *A. sauteri* were available, ants of *P. noda* were always observed

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Figure 10. Seasonal changes in the average numbers (with SE) of individuals of *Eumyrmococcus smithii* per colony and the age structure (percentage of components). Numerals above the bars indicate the sample size (presumed number of ant colonies). “Pupa” here includes pupae of both sexes and male prepupa, which were difficult to discriminate when not on slides.
foraging on the ground and were not found in the soil. Although nests of *T. biacarinatum* were found in the soil in the study plot, nests of *P. noda* were not (Table II).

### Discussion

**Growth stages of *Eumyrmococcus smithii***

The present study is the first to describe the female pupal stage of *E. smithii*. Within the family Pseudococcidae, the female adult stage normally follows three nymphal stages (Williams 1998). However, Williams (1988) described a female pupal stage for *Xenococcus* and argued that the pupal stage could be regarded as the third instar. Moreover, Williams (1998) found similar female pupal stages in females of at least five *Eumyrmococcus* species. Our study has shown that the morphology of the female pupa of *E. smithii* is very similar to that of congeneric species (Williams 1998). All mealybug species in intimate symbioses with *Acropyga* ants have thus far been found to have a non-feeding pupal stage, and thus our identification of a female pupal stage of *E. smithii* reinforces the association (pointed out by Williams 1998) between the formation of the female pupal stage and symbiosis with *Acropyga* ants.

We identified the morphology of first-instar nymphs for the first time in *Eumyrmococcus smithii* by scrutinizing the morphology of many individuals of *E. smithii*. Moreover, our study suggests that *E. smithii* has only one nymphal stage, which is followed by a pupal stage in females and by a prepupal stage in males (Figure 4). Williams (1998) argued that two nymphal stages would be expected for other species of *Eumyrmococcus*, and he reported that second-instar nymphs were available for 11 species, but described only that of *E. taylori* in detail and those of six other species very briefly. Williams (1998) also reported that no first-instar nymphs were available, except for a single individual still within its egg membrane. Our results suggest that the individual inside its egg membrane and the “second-instar”
nymphs described by Williams (1998) are the same instar. Rearing experiments will be necessary to more precisely describe the life cycle of *E. smithii*.

*Nest structure of Acropyga sauteri*

Our observations show that nests develop along the root systems of plants on which the mealybugs feed. As a consequence, the spatial borders between two neighbouring colonies cannot be simply mapped in a horizontal dimension. Using nestmate recognition methods to determine colony identity, we showed that colony boundaries often existed between two ant aggregations separated by only ca 5 cm and that the greatest distance between two aggregations that were judged to belong to the same colony was 25 cm. In addition, when the distance between two ant aggregations compared was greater than 30 cm, the aggregations were always found to belong to different colonies. These results suggest that a nest is restricted to a space of 0–30 cm, that an individual colony of *A. sauteri* may be densely concentrated in a small space around the root systems of plants of *M. sinensis*, and that colony boundaries can run diagonally, horizontally, and vertically through the root system.

Mapping ant distributions showed that the shortest distance observed between two queens was only 5 cm. This might cause problems with the determination of whether *A. sauteri* is monogynous or polygynous. As previously mentioned, however, our observation that many colonies were concentrated in a small space and the fact that multiple queens (except for new alate foundress queens) were not found within a single colony suggest that *A. sauteri* is monogynous. This suggestion is supported by Uye’s (1933) observation that *A. sauteri* colonies are founded by solitary queens. Without our tests of colony identity, the field situation could have been interpreted as a widely distributed polygynous nest.

*Seasonal changes in age structures for both partners*

On the basis of the following two observations, we inferred that the nuptial flight of *A. sauteri* mainly occurs from mid-March to early April on Okinawa Island. First, cross-seasonal comparisons of the numbers of reproductives in colonies that produced at least one reproductive showed that the number of reproductives per colony was rather high in March. Second, solitary queens unattended by workers were only observed in April. These queens were presumed to be solitarily founding their nests immediately after their nuptial flight. This inference agrees with Terayama’s (1988) observation of nuptial flights for this species in mid-March on Tokunoshima Island, near Okinawa Island. However, we found alate females and males in seasons other than March. Currently, we do not have sufficient information to judge whether these reproductives perform nuptial flights in seasons other than spring or whether they survive inside the nests until the following year’s nuptial flight. Uye’s (1933) observation of nuptial flights of *A. sauteri* in December in Oita Prefecture (to the north of Okinawa Island) supports the former possibility, although he regarded the observed phenomenon as an abnormal event.

The observed seasonal trends in numbers and age structures of the partners suggest that *E. smithii* reproduces mainly from April until August and that the total number of mealybug individuals decreases from October to March. These seasonal trends may be associated with seasonal fluctuations in temperature. Temperatures are usually under 20°C from November or December to March or April on Okinawa Island. The decrease in mealybug
numbers may be due to a low temperature, which is likely to negatively affect production activities (such as photosynthesis) by host plants. The seasonal trends in ant numbers also may be indirectly affected by temperature fluctuations. This is suggested by the fact that there was a lag period between fluctuations in ant numbers and fluctuations in mealybug numbers and by the reasonable inference that colony growth of *A. sauteri* depends on the reproduction of its symbiont, *E. smithii*. Delabie and Fowler (1993) reported that the population size of an *Acropyga* ant in symbiosis with a mealybug species showed significant lagged correlations with temperature, rainfall, and leaf flush. They proposed that the reason for the lagged correlations was the indirect dependence of the ant population on plants, via feeding on plant roots by the mutualistic mealybugs.

*Other ant species*

The observed frequent invasion of damaged nests by common ant species such as *P. noda* and *T. biacarinatum* suggests that nests of *A. sauteri* were normally well defended from ant attacks because of their almost completely subterranean lifestyle in certain kinds of soils. For nest sites, *A. sauteri* prefers rather hard clay layers lying underneath soft surface soil. By contrast, the other ant species located their nests mainly in the surface soil, usually at soil depths of 0–5 cm, which suggests that they may have difficulties digging in hard clay. The clay layer preferred by *A. sauteri* may function as a barrier to invasion by potential predators, such as the two ant species observed in this study.

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Appendix

Descriptions of the growth stages of Eumyrmococcus smithii Silvestri

Adult female. The adult female was described by Williams (1970, 1978, 1998).

Adult male (Figure 11). When alive, white, tip of abdominal curled ventrally, apterous. As mounted on slide elongate-pyrmiform, ca 1.1 mm in length \((n=1)\), ca 0.50 mm in width \((n=1)\) at cephalothorax, cephalothorax not dilated, ca 220.8 \(\mu\)m at base of abdominal segment \((n=1)\). Anal lobes, with one ventral seta and two short dorsal setae, stouter than body setae. Antennae situated on ventral side, 82.5–90.0 \(\mu\)m in length \((n=2)\), two-segmented, 55.2–66.7 \(\mu\)m \((n=2)\) in length of the second segment. Eyes, ostioles, and circulus absent. Legs stout, hind trochanter + femur 167.5–170.0 \(\mu\)m \((n=2)\), hind tibia + tarsus 127.5–132.5 \(\mu\)m \((n=2)\), claws ca 30.0 \(\mu\)m \((n=1)\). Ratio of lengths of hind tibia to tarsus 1.8–2.1. Labium absent. Spiracular opening 42.5–47.5 \(\mu\)m in diameter \((n=2)\). Genital capsule somewhat retracted inside abdomen. The penial sheath largely membranous. Ventrally, penial sheath cleft by a long narrow slit, protruded aedeagus with roundly pointed apex. In ventral view, sharply pointed in profile. Anus not visible. Dorsal and ventral surface of body with short setae only.

Comments: Williams (1998) described and illustrated adult males of two species belonging to Eumyrmococcus. This is the third description of an adult male for the genus Eumyrmococcus. Differing from E. sarawakensis Williams and Xenococcus species, the adult male of E. smithii has long and stout legs as in E. taylori Williams. However, the adult male of E. smithii does not have long anal lobe setae as seen in the adult male of E. taylori.

Female pupa (Figure 12a). As mounted on slide elongate-oval, 0.90–1.12 mm in length \((n=12)\), 0.60–0.78 mm in width \((n=12)\); cephalothorax not so dilated as female adult and nymph, tip of abdomen rounded. Antennae situated on ventral side, 142.5–177.5 \(\mu\)m in length \((n=16)\), two-segmented, apex rounded, each segment with short setae. Legs with distinct segmentation, with short setae, hind trochanter + femur 155.0–187.5 \(\mu\)m \((n=17)\), hind tibia + tarsus + claw 120.0–152.5 \(\mu\)m \((n=17)\). Labium distinct, 122.5–140.0 \(\mu\)m in length \((n=3)\), 69.0–124.2 \(\mu\)m in width \((n=15)\). Ratio of length to width of clypeolabral shield 0.78–1.09. Spiracle present, spiracular opening 28.8–35.0 \(\mu\)m in diameter \((n=17)\).

Male prepupa (Figure 12b). As mounted on slide elongate-oval, 1.05–1.90 mm in length \((n=14)\), 0.70–0.80 mm in width \((n=8)\) at cephalothorax, tip of abdomen rounded. Antennae situated on ventral side, 130.0–160.0 \(\mu\)m in length \((n=15)\), two-segmented, apex rounded, setae not distinct. Legs with distinct segmentation, with short setae, hind trochanter + femur 172.5–210.0 \(\mu\)m \((n=12)\), hind tibia + tarsus + claw 112.5–140.0 \(\mu\)m \((n=12)\). Labium remaining but not distinct. Spiracle present, spiracular opening 28.7–32.5 \(\mu\)m in diameter \((n=14)\).
Figure 11. Adult male, right side shows venter of the adult male; left side shows dorsum of the adult male. (a) Lateral view of genitalia of the adult male; (b) ventral view of genitalia of the adult male. Scale bar: 0.1 mm.
Figure 12. (a) Female pupa; (b) male prepupa; (c) male pupa. Right sides show venter of the prepupal or pupal stages; left sides show dorsum of the prepupal or pupal stages. Scale bars: 0.2 mm.
Figure 13. First-instar nymph, right side shows venter of the nymph; left side shows dorsum of the nymph. Anal lobe setae are long, but here only a part of the setae are drawn. Scale bar: 0.1 mm.
Male pupa (Figure 12c). As mounted on slide somewhat elongate-oval, 0.88–0.98 mm in length \((n=4)\), 0.46–0.80 mm in width \((n=5)\) at cephalothorax. Antennae situated on ventral side, 102.5–117.5 \(\mu\)m in length \((n=15)\), two-segmented, apex rounded, with short setae. Legs with distinct segmentation, with short setae, hind trochanter + femur 135.0–150.0 \(\mu\)m \((n=4)\), hind tibia + tarsus + claw 122.5–130.0 \(\mu\)m \((n=4)\). Labium absent. Spiracle present, spiracular opening 37.5–40.0 \(\mu\)m in diameter \((n=4)\).

First-instar nymph (Figure 13). When alive, white, tip of abdomen curled up dorsally, with cephalothorax dilated similar to female adult. Mounted on slide body elongate-pyriform, 0.72–1.39 mm in length \((n=83)\), 0.27–0.95 mm in width \((n=99)\); posterior end of body rounded and 78.2–184.0 \(\mu\)m at base of abdominal segment \((n=144)\). Anal lobes, with one ventral seta and two long setae, each about 350–460 \(\mu\)m in length. Antennae situated on ventral side, 145.0–182.5 \(\mu\)m in length \((n=174)\), two-segmented, 124.2–156.4 \(\mu\)m \((n=167)\) in length of the second segment. Eyes, ostioles and circulus absent. Legs stout, hind trochanter + femur 157.5–207.5 \(\mu\)m \((n=173)\), hind tibia + tarsus 132.5–177.5 \(\mu\)m \((n=175)\), claws slender, 47.5–55.0 \(\mu\)m in length \((n=25)\). Ratio of lengths of hind tibia to tarsus 1.3–2.7. Labium 97.5–125.0 \(\mu\)m in length \((n=170)\), 59.8–101.2 \(\mu\)m in width \((n=108)\). Ratio of length to width of clypeolabral shield 0.8–1.3. Spiracular opening 20.0–25.0 \(\mu\)m in diameter \((n=181)\). Anal ring 40.0–52.5 \(\mu\)m in width \((n=74)\), with six to eight setae; the shortest in anterior seta ca 18.4 \(\mu\)m, the longest in posterior seta ca 414 \(\mu\)m. Flagellate setae rather densely covering body but relatively sparser than those of female adult; long dorsal setae on cephalothorax about 30–62 \(\mu\)m in length, short dorsal setae on cephalothorax about 8–22 \(\mu\)m in length; long dorsal setae on abdomen about 45–82 \(\mu\)m in length, short dorsal setae on abdomen about 12–32 \(\mu\)m in length; ventral setae on cephalothorax longer and stouter than those on dorsal side; ventral abdominal setae same size or slightly longer than those on dorsal side.

Material examined

All specimens were collected on Sashiki Town, Okinawa Island, Japan, on the roots of Miscanthus sinensis Anderss in the nests of the ant species Acropyga sauteri Forel. The specimens were collected on 4 April 2000 (K. Kishimoto-Yamada) and on 29 April 2000 (H. Takamine). The voucher specimens are deposited in the Kyoto University Museum (KUM) [Eumyrmococcus smithii Silvestri, two adult females, two adult males, two female pupae, two male pupae, two male prepupae, seven first-instar nymphs, two female pupae with adult exoskeletons, two male pupae with adult exoskeletons (Kishimoto-Yamada’s code KKY1–21); Acropyga sauteri Forel, two workers, one queen (KKY22–24)].