ORIGINAL ARTICLE

Reproduction site preference and performance by sexuparae, and mating behavior of their sexual generation on the primary host plant in a heteroecious aphid, *Neothoracaphis yanonis* (Homoptera: Aphididae)

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Abstract

We studied reproduction site preference and performance by the sexuparae (autumnal migrants) of *Neothoracaphis yanonis* and mating behavior of their sexual generation on its primary host plant, *Distylium racemosum*. The sexuparae preferred younger leaves of *D. racemosum* for settlement and imbibing leaf sap, and they produced more offspring there than on older leaves. Thus, it is suggested that the sexuparae selected more nutritious younger leaves to increase their own fecundity. The offspring consist of yellowish dark-grey and creamy yellow type nymphs, which develop into small males and large oviparae, respectively. Yellowish dark-grey nymphs were deposited gregariously on the basal part of the abaxial surface of a leaf blade, while creamy yellow nymphs were deposited evenly over the abaxial surface. Such a localized distribution pattern probably resulted from sexuparae's strategy to enhance a possibility for their male offspring to mate with oviparae at the base of leaf blade, over which oviparae crawl to twigs. During mating and sometimes afterwards, the ovipara incidentally carried a male on her back and crawled downward on twig to find an axillary bud for oviposition. This behavior may be advantageous to males, who can guard their mate during this period against other conspecific males. Such mate-guarding behavior seems to be related to the development of dual mate-seeking strategies, in which males try to copulate, first at the basal part of midrib, and second on the axillary bud.

Key words: apterous, axillary bud, dual mate-seeking strategies, gall aphid, sex ratio.

INTRODUCTION

In relation to intraplant heterogeneity of nutrient availability over a young developing leaf blade, some studies have demonstrated that fundatrices (foundress) of gall aphids exhibit a preference of galling site to achieve their maximum fitness (e.g. Whitham 1978; Busenberg & Velasco-Hernandes 1994). Fundatrices of the heteroecious (host-alternating) gall aphid *Neothoracaphis yanonis* (Matsumura) (Homoptera: Aphididae: Hormaphidinae),

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for example, prefer the basal intermediate part of a newly developing leaf blade of their primary host plant, *Distylium racemosum* (Siebold & Zuccarini) (Hamamelidaceae) and on this part they induce larger galls containing more numerous alate offspring (Ngakan & Yukawa 1996). From late April to early May, alates of *N. yanonis* emerge from the galls and migrate to the secondary host plant, *Quercus serrata* Thunberg or *Q. crispula* Blume (Fagaceae) and alate sexuparae (autumnal migrants) return to *D. racemosum* in late November (Monzen 1934; Moritsu 1983).

Sexuparae of heteroecious gall aphids of the subfamilies of Hormaphidinae and Eriosomatinae, including *N. yanonis*, settle on their primary host plant later in the season and parthenogenetically produce offspring consisting of males and oviparae. Usually the phloem

sap of mature leaves is less nutritious than that of young leaves (Hikosaka *et al*. 1994), exhibiting marked seasonal changes in the quality of food available to aphids (Dixon 1985). In order to test whether sexuparae of heteroecious aphids exhibit reproduction site preference and performance, *N. yanonis* is one of the most appropriate study materials. This is because sexuparae of *N. yanonis* settle on mature leaves of *D. racemosum*, an evergreen tree and hence the leaves can be distinguished into different categories according to the age of shoots.

Aphid mating behavior has been described in various papers (e.g. Steffan 1990; Foster & Benton 1992; Guldemond *et al*. 1994; Guldemond & Dixon 1994; Doherty & Hales 2002; Huang & Caillaud 2012). Some earlier studies have focused on the ability of males to recognize pheromone released by conspecific or congeneric oviparae or gynoparae (Hardie *et al*. 1996; Galli 1998). In the subfamily Aphidinae, heteroecious aphids have winged males (Dixon 1985), which are able to fly directly to pheromone-releasing oviparae. However, in eriosomatine and hormaphidine aphids, alate sexuparae produce apterous males and oviparae, and thus apterous males cannot approach oviparae in such a manner. Other studies have addressed various aspects of mating behavior such as correlation between sex ratio and mate competition or polygamy (Kozlowski 1991; Foster & Benton 1992), correlation between age or body size and mating success (Doherty & Hales 2002; Hales 2005), and the avoidance of inbreeding by recognizing close kin (Huang & Caillaud 2012). Different aphid subfamilies and other taxa exhibit different mating behavior because the life cycle of aphids is unique and greatly varies with subfamilies and other taxa. Therefore, many other aspects of mating behavior, especially those related to the larviposition tactics of sexuparae or gynoparae, remain unclear.

The purposes of this paper are to (i) test if sexuparae of *N. yanonis* exhibit reproduction site preference by comparing the percentage of leaves settled by sexuparae and the mean number of sexuparae per leaf between different leaf age categories; (ii) in relation to the preference of sexuparae, evaluate performance by the sexuparae by comparing the mean number of their sexual offspring between different leaf age categories; and (iii) clarify spatial distribution patterns of apterous males and oviparae on a host leaf blade. We also describe detailed mating behavior of the males and oviparae in terms of their spatial distribution patterns on the host leaves and dual mate-seeking strategies.

MATERIALS AND METHODS

The aphid

The life cycle of *N. yanonis* has been described on many occasions (e.g. Monzen 1934; Yamaguchi 1954; Moritsu 1983; Yukawa & Masuda 1996; Blackman & Eastop 2020). We review its life cycle in relation to the present study as follows. In southern Kyushu, Japan, *N. yanonis* fundatrices start to induce small, prominent galls protruding from both sides of leaves of its primary host plant, *D. racemosum*, from late February to early March. Galls become mature in late April and contain up to 105 offspring (depending on gall size) derived from each fundatrix (Ngakan & Yukawa 1996). Galls dehisce from late April to early May, and alate offspring that emerge from the galls migrate to secondary host plants such as *Q. serrata* or *Q. crispula*. In the middle to late November, sexuparae return to *D. racemosum* and fly from leaf to leaf in search for an appropriate one for reproduction. Once a sexupara selects an appropriate leaf, it remains there and imbibes leaf sap. About 4 to 6 days later, it starts to produce nymphs one by one on the abaxial surface of the leaf. During the reproduction period, sexuparae continuously imbibe leaf sap and it was sometimes observed that they changed their position on leaves. The offspring of sexuparae consist of yellowish dark-grey type (hereafter YDG-type) nymphs and creamy yellow type (hereafter CY-type) nymphs (e.g. Moritsu 1983), which later develop into small males and large oviparae, respectively. The body length of the YDG-type nymphs is about 0.41 mm and that of the CY-type ones is about 0.33 mm. Field observations showed that nymphs of both types imbibe leaf sap from the abaxial surface of host leaves, unlike nymphs of eriosomatine aphids that do not (Heie 1980; Foster & Benton 1992). Both types of offspring did not move actively over the leaf blade before they reached adulthood. In late December to early January, the YDG-type nymphs develop into a small (about 0.52 mm in body length) but highly agile adult males, and the CY-type nymphs develop into large adult oviparae, which are about 1.12 mm in body length. After mating, oviparae lay up to 16 eggs usually in crevices around axillary buds. Fundatrices hatch from overwintered eggs from late February to early March, move to fresh leaves, and start to induce galls on the leaves.

Field surveys

Field surveys were conducted from November 1995 to February 1996 for several mature trees (about 6 m in

height) of *D. racemosum* in the arboretum of Kagoshima University, Kagoshima City, southern Japan. To determine whether sexuparae prefer younger to older leaves for settlement to produce sexual offspring, the number of sexuparae was recorded in late November 1995 for a total of 528 randomly sampled leaves attached to shoots of three different age categories: (i) 176 leaves on shoots of the previous year (including spring and lammas shoots in 1994; hereafter 1-year old shoots); (ii) 176 leaves on spring shoots of current year (hereafter spring shoots); and (iii) 176 leaves on lammas shoots of current year (emerged in late summer of 1995; hereafter lammas shoots). Between the three categories, the percentages of leaves settled by sexuparae were compared using χ^2 test, and the mean numbers of sexuparae per leaf were compared using Tukey's HSD test.

To compare the performance between sexuparae settled on different age categories of leaves, a week later, 88 leaves were sampled, respectively, for each age category. To avoid confounding effects of density, and to ensure that offspring were produced by one sexupara, sampled leaves were restricted to those with a single sexupara. The number of offspring per leaf was recorded, and the mean numbers of offspring per leaf were compared using Tukey's HSD test.

Because offspring do not move actively over the leaf blade before they reach adulthood, the distribution pattern of males and oviparae on a leaf blade reflects the strategy of sexuparae in producing offspring. To clarify reproduction site preference within a leaf blade by sexuparae, a leaf blade was imaginarily divided into four parts from base to apex: base, where a petiole is located (hereafter A-part), intermediate basal part (hereafter B-part), intermediate apical part (hereafter C-part), and apical part (hereafter D-part), as shown in Ngakan and Yukawa (1996, 1997). In early December 1995 when both males and oviparae were in the 1st or 2nd stadium, their number on the four leaf parts was recorded for 130 leaves (40 leaves on 1-year old shoots, 60 leaves on spring shoots, and 30 leaves on lammas shoots) on which offspring were found. Then, the observed relative abundances of males and oviparae on each leaf part were compared, respectively, with those expected from even distribution (25% : 25% : 25% : 25%) using χ^2 test.

The numbers of males and oviparae on the 130 leaves observed above were counted, and the mean numbers of males and oviparae per leaf were compared among leaf age categories using Tukey's HSD test. The sex ratio (the proportion of male to oviparae) was calculated for each leaf and the mean sex ratios among leaf age categories were compared using Tukey's HSD

test. All statistical analyses were performed using R version 3.6.1 (R Core Team 2019).

Observations of mating behavior

Mating behavior of males and oviparae was observed in the laboratory of Entomology, Faculty of Agriculture, Kagoshima University, Kagoshima City from late December 1995 to mid-January 1996. Ten twigs with leaves inhabited by late stadium males and oviparae were collected from the census tree, and each twig was held in water in a bottle to keep it fresh and then placed on a table at room temperature. When offspring became adult males and oviparae, their mating behavior on the 10 twig samples was observed under a dissecting microscope daily between 01:00 PM to 6:00 PM, since a preliminary observation revealed that mating occurred between 4:00 PM and 5:00 PM. Observations on the 10 twig samples continued until all the oviparae died. Using material sampled from the campus of Kyusyu University, Fukuoka City, additional observations were made in December 2001 and 2003 in the Entomological Laboratory, Faculty of Agriculture, Kyushu University, Fukuoka City to obtain photograph data.

RESULTS

Reproduction site preference by sexuparae

In late November 1995, sexuparae flew back to *D. racemosum* trees, settled on the abaxial surface of host leaves and started imbibing (Fig. 1a). The percentage of leaves settled by sexuparae was significantly larger on spring and lammas shoots than on 1-year old shoots (Table 1). The mean number of sexuparae per leaf was significantly greater on the leaves of spring and lammas shoots than those of 1-year old shoots (Table 1). These data indicate that sexuparae prefer younger leaves for settlement.

Performance by sexuparae in the production of offspring

On leaf samples occupied by a single sexupara, the mean number of offspring (YDG- and CY-type nymphs were taken together) per leaf on spring or lammas shoots was significantly greater than that on 1-year old shoots (Table 2). The maximum number of offspring on a single leaf was 15 on spring shoots and the minimum number was zero. This indicates that a few sexuparae have just arrived on the leaf and have not yet begun to produce offspring.
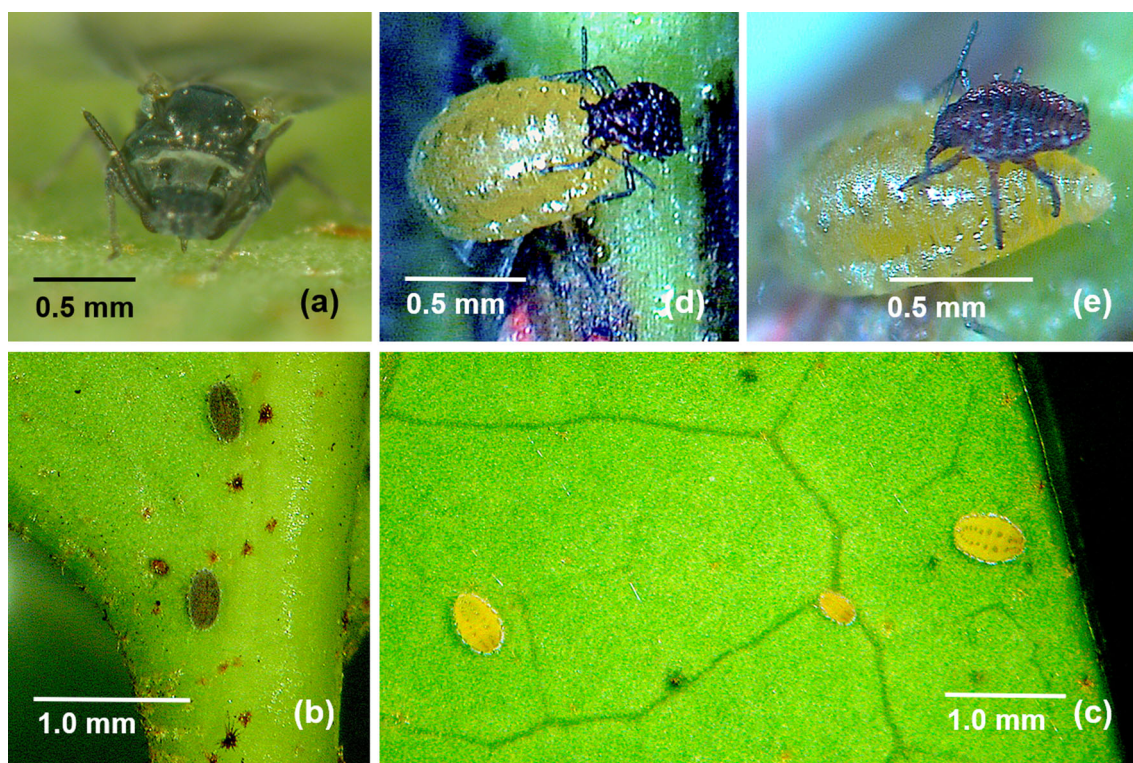
Figure 1 (a) A sexupara imbibes leaf sap while settled on abaxial surface of *Distylium racemosum* leaf; (b) males distributed gregariously almost at the conjunction between leaf blade and petiole; (c) oviparae distributed evenly over a leaf blade; (d) a male mating an ovipara; (e) a male does not dismount from an ovipara immediately after mating.

Table 1 Comparison between leaf age categories in the percentage of leaves settled by sexuparae of *Neothoracaphis yanonis* and the mean number of sexuparae per leaf

	Leaves on different age categories of shoots		
	1-Year shoot	Spring shoot	Lammas shoot
No. of leaves examined	176	176	176
No. of leaves with sexuparae	43	91	83
% of leaves with sexuparae	24.43 a	51.71 b	47.16 b
Mean no. of sexuparae/leaf	0.26 (±0.046) y	0.61 (±0.046) z	0.57 (±0.046) z

Different alphabetical letters after percentages and means indicate significant differences (χ^2 = 31.045, d.f. = 2, $P < 0.05$, follow-upped using pairwise Chi-squared test with Bonferroni method, $P < 0.05$ for percentages of leaves settled by sexuparae; Turkey's HSD test, $P < 0.05$ for mean number of sexuparae per leaf); the values after ± indicate standard error.

Table 2 Comparison of the mean number of offspring produced by sexuparae of *N. yanonis* on leaves of different age categories

	Leaves on different age categories of shoots		
	1-Year shoot	Spring shoot	Lammas shoot
No. of leaves examined	88	88	88
Total no. of offspring	41	140	139
Mean no. of offspring/leaf	0.47 (±0.24) a	1.59 (±0.24) b	1.58 (±0.24) b

Different alphabetical letters after means indicate significant differences (Turkey's HSD test, $P < 0.05$); the values after ± indicate standard error.

A total of 1,054 offspring (males + oviparae) was found on 130 leaves of the three shoot categories surveyed (Table 3). The mean number of males per leaf was not significantly different between different shoot categories. The mean number of oviparae per leaf was significantly greater for the leaves of spring and lammas shoots than for those of 1-year old shoots. Mean sex ratio was not significantly different between leaves on different shoot categories. Mean percentage sex ratio for the total of 130 leaves (leaves of all age categories were taken together) was 26.69 (± 3.46 SE).

Spatial distribution patterns of males and oviparae on a leaf blade

The spatial distribution patterns of both males and oviparae on a leaf blade are shown in Fig. 2. A large majority of males were found on the A-part and followed by the B-, C-, and D-parts (see also Fig. 1b). This pattern was apparently gregarious, and significantly different from the even distribution pattern (Fig. 2). In contrast, the distribution pattern of oviparae

was not significantly different from an even distribution (see also Fig. 1c).

Mating behavior

YDG- and CY-type nymphs (offspring of sexuparae) developed respectively into males and oviparae almost simultaneously within 3 to 4 weeks. Then, oviparae left leaf parts where they had settled during development and crawled slowly towards the petiole to move onto a twig. Males, most of which had been located on the A-part of a leaf blade, gathered at the basal part of the midrib (around the conjunction between the base of leaf and petiole, see Fig. 1b) to wait for oviparae. When both sexes encountered, the male mounted the ovipara for mating. Because the number of oviparae was greater than that of males, every male mated with an ovipara on the base of the midrib. In contrast, oviparae that failed to mate with a male at the base of the midrib keep crawling downward to the twig.

While the male was trying to locate an appropriate position for mating, the ovipara continuously crawled downward on the twig carrying the male on her back.

Table 3 The sex ratio of offspring (males and oviparae) produced by sexuparae of *Neothoracaphis yanonis* on 130 leaves of different age categories

	Leaves on different age categories of shoots		
	1-Year shoot	Spring shoot	Lammas shoot
No. of leaves examined	40	60	30
Total no. of males	43	76	49
Total no. of oviparae	203	406	277
Mean no. of males/leaf	1.08 (±0.22) a	1.27 (±0.18) a	1.63 (±0.76) a
Mean no. of oviparae/leaf	5.08 (±0.70) b	6.77 (±0.57) c	9.23 (±0.81) c
Mean sex ratio (%)	26.71 (±6.26) d	24.36 (±5.11) d	31.27 (±7.23) d

Different alphabetical letters after means indicate significant differences (Turkey's HSD test, $P < 0.05$); the values after ± indicate standard error.
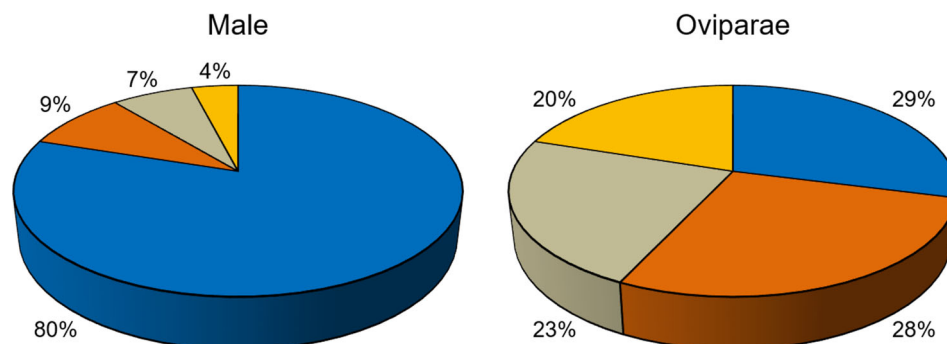

Figure 2 The spatial distribution pattern of males and oviparae of *Neothoracaphis yanonis* on the four parts of a leaf blade (from base to apex: A-, B-, C-, and D-parts). The distribution pattern of males was significantly different from the even distribution pattern (25% : 25% : 25% : 25%) ($\chi^2 = 61.671$, d.f. = 3, $P < 0.05$), but that of oviparae was not significantly different ($\chi^2 = 1.105$, d.f. = 3, $P = 0.776$).

When the mating started after the male located an appropriate position, the ovipara stopped crawling (Fig. 1d). Mating continued 2 to 2.5 min. After mating, the male stayed on the back while the ovipara crawled on a twig to find a suitable site for oviposition (Fig. 1e), which was usually in crevices around axillary buds. The longest record of carrying time was about 40 min.

When an ovipara carrying a male is passing near an axillary bud where there are other males, the free male that had dismounted from another ovipara after completion of mating and remained around the axillary bud tried to mount the ovipara. Then fighting occurred between the two males. The intruder climbed onto the former males on the back of ovipara and tried to pry the male off using his hind legs. We did not observe specialized fighting morphology, but the intruder usually failed to displace the first male. When oviparae were not present nearby, fighting never occurred among males either on twigs near axillary buds or even on the basal part of leaf blade.

After leaving the ovipara, the male remained around axillary buds, which were the most preferred oviposition site, to wait for other oviparae to arrive regardless of whether they were virgin or not. Because of the female-biased sex ratio (Table 3), most oviparae could not meet with males at the basal part of midrib. Such virgin oviparae crawled down on the twig to axillary buds, where they could encounter males and sometimes mate with them. Some oviparae were observed to mate twice with different males; first at the basal part of midrib and the second near an axillary bud. Rarely the same couple mated twice. Thus, multiple copulations became evident for both sexes in this study. Within 24 h after mating, oviparae began to lay their eggs. Oviparae died within 24 h after laying eggs, while males survived for a few days longer.

Oviparae became wrinkled, listless, and darker in color 2 days after emergence. No males were observed to mate with aged oviparae, even though they were virgin. Some oviparae never encountered males, and failed to oviposit.

DISCUSSION

It has long been suggested that older leaves usually cannot support higher growth or fitness of herbivores than younger leaves presumably due to the decrease in nutrients and an increase in the concentration of chemical defences (Kennedy et al. 1950; Feeny 1970; Reynolds & Smith 1985; Hikosaka et al. 1994; Cao et al. 2018). The present study also revealed that sexuparae of N. yanonis preferred younger leaves for settlement and imbibed leaf sap on the leaves they settled (Fig. 1a, Table 1). As a result, they could produce more offspring on younger leaves than on older ones (Tables 1, 2). The selection of younger leaves by sexuparae may also provide nutritious diet for offspring (males and oviparae), because the offspring are immobile before developing into adults.

An opposite behavior, however, has been reported by Glinwood and Pettersson (2000) to occur on the heteroecious bird cherry-oat aphid, Rhopalosiphum padi (Linnaeus) (Homoptera: Aphididae: Aphidinae). Gynoparae (autumnal migrants) of the aphids selected leaves irrespective of senescence stage when they return to the primary host plant, Prunus padus (Linnaeus) (Rosaceae) in autumn. Instead, oviparae produced by gynoparae were mobile and had the capacity to evaluate leaf senescence for their survival and fitness. The nutrient contents of P. padus leaves may not be significantly different between different stages of senescence, because they appear simultaneously from the overwintered buds early in spring and all will be shed during autumn (King 2005). Glinwood and Pettersson (2000) pointed out that such behavior may have evolved because the leaf fall would be a definite deadline for reproduction. Unlike deciduous P. padus, D. racemosum is an evergreen tree and at least three different age categories of leaves are available for sexuparae of N. yanonis. Feeding on the host leaves before production of offspring might be the reason why they showed preference for young leaves. In addition, males and oviparae do not necessarily move actively, because most of them were born on the younger leaves that were still nutritious.

It was remarkable that a large majority of males was found on the most basal part (A-part) of the leaf blade, while oviparae were distributed almost evenly among the four parts from base to apex of a leaf blade (Fig. 2). This spatial distribution pattern of oviparae suggests even partitioning of nutrients over a leaf blade, because in the mature leaves the development of fibre and nutritional content had attenuated (Agnusdei et al. 2012). Therefore, the waiting of males on the A-part (particularly on the connection between petiole and leaflet; Fig. 1b) is not for obtaining more nutrients, as has been noted for gall-inducing fundatrices of N. yanonis (Ngakan & Yukawa 1996), but is an ideal behavior for apterous males to enhance the possibility of encountering oviparae which move through the basal portion down to a twig.

Males of N. yanonis have dual mate-seeking strategies in which males try to copulate first at the basal part of midrib, and second on the axillary buds. The strategies are efficient for apterous males as an

alternative to the mating system using oviparae-releasing pheromone (Hardie *et al.* 1996). Such dual mate-seeking strategies have been noted in male bees such as *Centris pallida* Fox (Hymenoptera: Anthophoridae) (Alcock *et al.* 1977) and *Osmia rufa* (Linnaeus) (Hymenoptera: Megachilidae) (Raw 1976), and in many syrphid species (Diptera) (Maier & Waldbauer 1979). However, such mate-seeking strategies have not been described for aphids.

Even after completion of mating, males did not get off the oviparae immediately (Fig. 1e). Oviparae carrying males on their back crawled downward on twigs to find axillary buds for oviposition. Such male behavior has been reported for *Myzus persicae* (Sulzer) (Homoptera: Aphididae) (Doherty & Hales 2002) and for *Pemphigus spyrothecae* Passerini (Homoptera: Aphididae) (Foster & Benton 1992). The dual mate-seeking strategies may have evolved in relation to multiple copulations. This behavior is advantageous to males, who can guard their mate during this period against other conspecific males.

Although the sex ratio of the offspring is largely biased towards females (Table 3), competition never occurs between females (oviparae). In contrast, we sometime observed male–male fighting. Such moderate male–male competition is similar to that revealed by Foster and Benton (1992) in *P. spyrothecae* and by Akimoto and Yamaguchi (1997) in *T. sorini*. Fighting among males in the condition of a largely female-biased sex ratio may be due to locally high density of males; the oviparae actively move, while males do not actively seek for oviparae, but gather around axillary buds to wait for an ovipara passing through. Therefore, when an ovipara (regardless of whether she carries a male or not) passes close to an axillary bud, males will race each other to ride the ovipara. Without attendance of an ovipara, male–male fighting was never observed, and rather two or more males coexisted peacefully around an axillary bud. Actively seeking for oviparae could be disadvantageous to males rather than waiting for them on the axillary buds.

Up to this point, this study has successfully revealed that, similar to the fundatrices when selecting a site to produce gall on a fresh developing-leaves (Ngakan & Yukawa 1996), sexuparae of *N. yanonis* also showed preference for younger leaves, where they can produce more offspring. In addition, this study has successfully revealed the mating behavior of sexual offspring, especially the strategies that are carried out by wingless males and oviparae to encounter each other. However, this study still leaves some unanswered questions: whether or not an ovipara that feed on the sap of younger leaves can produce more eggs; what is the maximum mating frequency for an ovipara and a male; whether or not mating frequency affects the number of eggs an ovipara can produce; and whether or not oviparae select oviposition site optimal to fundatrices that hatch from the eggs. More intensive observations are required to answer these questions.

ACKNOWLEDGMENTS

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