

Research Article

Defensive Nymphs of the Woolly Aphid *Thoracaphis kashifolia* (Hemiptera) on the Oak *Quercus glauca*

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Aphid nymphs with enlarged fore- and mid-legs were found from woolly colonies of *Thoracaphis kashifolia* (Hormaphidinae, Nipponaphidini) on leaves of the evergreen *Quercus glauca* in Japan. It was shown that they grasped an introduced moth larva with their legs and some inserted their stylets deep into the body. These defenders were first-instar nymphs of the alate generation and were produced by aleyrodiform apterae from early September onward. There was a large variation in the size of their forelegs. First-instar nymphs (to be alates) produced early in the season had fore-femorotrochanters shorter than those produced later. The molting rate (the percentage of pharate individuals) of the latter was very low (less than 5% to zero), suggesting their semisterility. Although first-instar nymphs with various lengths of forelegs joined to attack moth larvae, these facts indicate that an incipient caste differentiation occurs within the first-instar nymphs of the alate generation.

1. Introduction

Since the discovery of aphid soldiers in *Colophina clematis* [1], sterile or nonsterile defensive nymphs have been found in many species of two aphid subfamilies, Eriosomatinae and Hormaphidinae [2–5]. The former subfamily consists of three tribes, Eriosomatini, Pemphigini, and Fordini, and the latter also consists of three, Hormaphidini, Cerataphidini, and Nipponaphidini [6, 7]. These aphid species basically have a host-alternating life cycle; most of them induce galls on their primary host and form exposed colonies on their secondary host. Defensive individuals (usually first- or second-instar nymphs [2] but at times fourth-instar nymphs [8] or apterous adults [9, 10]) have been recorded from the gall or the primary-host generations of all six tribes [9–17], and from the secondary-host generations of Eriosomatini [1], Cerataphidini [15, 18–21], and Hormaphidini [22]. Although exules of *Paracletus cimiciformis* (Fordini) have recently been shown to suck on ant larva hemolymph [23], defensive behavior on the

secondary host has been unknown from the remaining three tribes to date.

In the course of studying the life cycle of the aphid *Thoracaphis kashifolia* (Nipponaphidini) in Japan, we noticed soldier-like first-instar nymphs with thickened fore- and mid-legs in its woolly colonies on leaves of the evergreen oak *Quercus glauca*. Having introduced lepidopteran larvae onto the colonies, we succeeded in inducing defensive behavior by these nymphs. In this paper, we describe the life cycle of *T. kashifolia*, the defensive behavior of the first-instar nymphs and when they are produced in the life cycle. Because there was a large variation in the size of their forelegs, we address the issue whether soldier-caste differentiation occurs in this species.

2. Materials and Methods

2.1. Study Organism. The aphid *Thoracaphis kashifolia* (Uye) (the species epithet has been misspelled as “*kashifoliae*”



FIGURE 1: (a) Colonies of *Thoracaphis kashifolia* on leaves of *Quercus glauca* (Tama, Tokyo; 14 October 2015); (b) a woolly colony of *T. kashifolia* (Tama, Tokyo; 14 October 2015); (c) two apterous adults and one nymph to be aptera (upper right) of *T. kashifolia* (Tama, Tokyo; 8 February 2016); (d) defensive nymphs of *T. kashifolia* clinging to an experimentally introduced lepidopteran larva (Tama, Tokyo; 1 October 2015).

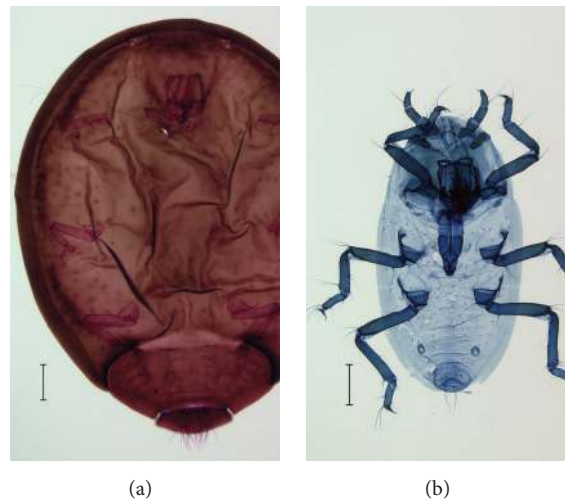


FIGURE 2: Apterous generation of *Thoracaphis kashifolia* (Ome, Tokyo; 6 November 2013): (a) adult; (b) first-instar nymph. Scale bars: 100 μm .

[6, 24, 25] or “*kashiwae*” [26–28]) forms colonies on the upper surfaces of leaves of *Quercus glauca* (Figures 1(a) and 1(b)) in the south-western half of Japan [27, 28] and in Taiwan [24]. A record from *Q. acuta* [29] remains to be confirmed. The apterous adults are aleyrodiform (Figures 1(c) and 2(a)), sessile, and flattened and are found throughout the year on the leaves

[27]. The colonies produce alates in autumn and become remarkably woolly like candy floss (Figure 1(b)) during this period. The life cycle is supposed to be anholocyclic, without returning to *Distylium racemosum* [6, 27], which is the only known primary host of Nipponaphidini in Japan [30–32]. In this paper, colonies that are covered with long wax filaments

TABLE 1: Collection data, the number of nymphs to be alates, the percentage of first-instar nymphs and their molting rate, and the most advanced stage of the alate generation for 23 woolly colonies.

Colony code	Collection place	Date of collection [†]	Number of nymphs to be alates [‡]	% first-instar nymphs to be alates [§]	Most advanced stage of the alate generation
15226	Tama, Tokyo	4 Sep. 2015	616 (722)	90.2 [§] (27.5) [§]	2nd instar
15228	Tama, Tokyo	12 Sep. 2015	174 (265)	99.3 [§] (16.8) [§]	2nd instar
15230	Tama, Tokyo	20 Sep. 2015	252 (315)	61.1 (9.1)	3rd instar
Exp#1	Tama, Tokyo	28 Sep. 2015 (29 Sep)	425 (532)	32.9 (1.4)	4th instar
Exp#2	Tama, Tokyo	28 Sep. 2015 (29 Sep)	922 (1150)	40.6 (2.3) [§]	4th instar
Exp#3	Tama, Tokyo	28 Sep. 2015 (29 Sep)	460 (532)	27.4 (0.8)	4th instar
Exp#4	Tama, Tokyo	30 Sep. 2015 (1 Oct)	427 (549)	35.4 (1.3)	Adult
Exp#5	Tama, Tokyo	30 Sep. 2015 (1 Oct)	1203 (1483)	37.8 (1.8) [§]	Adult
Exp#6	Tama, Tokyo	30 Sep. 2015 (1 Oct)	442 (568)	42.8 (4.8)	4th instar
Exp#7	Tama, Tokyo	30 Sep. 2015 (1 Oct)	548 (651)	40.9 (2.2)	4th instar
15241	Kyoto, Kyoto Pref.	5 Oct. 2015	160 (257)	31.3 (2.0)	Adult
14158	Tama, Tokyo	8 Oct. 2014 (10 Oct)	218 (377)	11.0 (0)	Adult
14160	Tama, Tokyo	8 Oct. 2014 (10 Oct)	240 (618)	56.7 (0)	Adult
15257	Tama, Tokyo	13 Oct. 2015	210 (255)	21.9 (4.3)	4th instar ^{††}
15258	Tama, Tokyo	13 Oct. 2015	258 (371)	24.0 (0)	Adult
15260	Tama, Tokyo	13 Oct. 2015	172 (267)	25.0 (2.3)	Adult
UE1013	Tsuchiura, Ibaraki Pref.	13 Oct. 2015	88 (180)	1.1 (0)	Adult
14167	Hachioji, Tokyo	26 Oct. 2014	81 (254)	6.2 (0)	Adult
14168	Hachioji, Tokyo	26 Oct. 2014	25 (92)	0	Adult
15271	Tama, Tokyo	27 Oct. 2015	85 (165)	1.2 (0)	Adult
15272	Tama, Tokyo	27 Oct. 2015	43 (112)	0	Adult
15273	Tama, Tokyo	27 Oct. 2015	74 (198)	1.4 (0)	Adult
15274	Tama, Tokyo	27 Oct. 2015	76 (157)	2.6 (0)	Adult

[†]Date of fixation in parenthesis. [‡]Colony size in parenthesis. [§]Percentage to all nymphs to be alates, with the molting rate (%) of first-instar nymphs in parentheses. [§]Estimated from a subsample. ^{††}Alate adults had probably been produced in this colony.

(Figure 1(b)) are called “woolly colonies,” while those that are not are “ordinary colonies” (colonies in Figure 1(a) but the central woolly colony).

2.2. Sampling of the Aphids. To investigate the annual life cycle and the colony composition, *T. kashifolia* was sampled from *Quercus glauca* in various months. We regarded the aphids on a single leaf as a colony and sampled colonies mainly in Tama, Hachioji and Ome, western Tokyo, Japan, in 2013–2016. Additional colonies were sampled in Tsuchiura (Ibaraki Prefecture), Kashihara (Nara Prefecture), and Kyoto (Kyoto Prefecture) in 2015. The whole colonies each were preserved in a 30 mL glass vial of 80% ethanol together with the leaf. Later, under a dissecting microscope in the laboratory, the aphids in 23 woolly colonies listed in Table 1 and 22 ordinary colonies in Table 2 were detached from the leaf, counted, and sorted into the following groups: (1) apterous adults, (2) first-instar nymphs to be apterae, (3) non-first-instar nymphs to be apterae, (4) alate adults, (5) first-instar nymphs to be alates, and (6) non-first-instar nymphs to be alates.

From some woolly colonies, emerged alates were collected and used for transfer experiments to confirm whether

these alates are sexuparae or secondary migrants (Section 2.5). For the same purpose, some alates (22 alates collected in Tama on 30 September 2015, nine in Tama on 15 October 2014, and 12 in Ome on 6 November 2013) were confined, together with a piece of paper, in a 5 mL cotton-plugged glass vial to force their larviposition there. A few days later, after confirming first-instar nymphs walking in the vial, 80% ethanol was poured into it. These nymphs were slide-mounted (see Section 2.3), and it was determined whether they were of sexuals or virginoparae (i.e., whether their mothers were sexuparae or secondary migrants). To supplement this, ten alates from colony 15241 and ten alates from colony UE1013 (Table 1) were slide-mounted, and it was determined whether the embryos in their bodies were the same in morphology as the first-instar nymphs born in the glass vial.

2.3. Examination of Aphid Morphology. For slide preparation, aphids preserved in 80% ethanol were cleared in heated 10% KOH solution. These aphids were stained with either Evans' blue or acid fuchsine, dehydrated in a mixture of glacial acetic acid and methyl salicylate for one day, and mounted in balsam via a mixture of xylo-phenol and pure xylo.

TABLE 2: Collection data, the number of apterous adults and those of nymphs for 22 ordinary colonies.

Colony code	Collection place	Date of collection	Number of apterous adults	Number of nymphs to be apterae	Number of nymphs to be alates
15001	Tama, Tokyo	3 Jan, 2015	7	2	0
15003	Tama, Tokyo	3 Jan, 2015	3	5	0
16004	Tama, Tokyo	8 Feb, 2016	52	6	0
16006	Tama, Tokyo	8 Feb, 2016	13	1	0
15022	Tama, Tokyo	3 Mar, 2015	18	2	0
15119	Tama, Tokyo	21 Apr, 2015	6	4 [‡]	0
15144	Kashihara, Nara Pref.	19 May 2015	7	42	0
15208	Tama, Tokyo	17 Jun, 2015	30	31	0
15218	Tama, Tokyo	27 Jul, 2015	56	17	0
15221	Tama, Tokyo	19 Aug, 2015	64	1 [†]	0
15223	Tama, Tokyo	19 Aug, 2015	95	1 [†]	0
15236	Tama, Tokyo	30 Sep, 2015	0	6 [‡]	0
15237	Tama, Tokyo	30 Sep, 2015	0	4 [‡]	0
15238	Tama, Tokyo	30 Sep, 2015	0	25 [‡]	0
15239	Tama, Tokyo	30 Sep, 2015	0	23 [‡]	0
14170	Tama, Tokyo	26 Oct, 2014	1 [†]	52	0
14171	Tama, Tokyo	26 Oct, 2014	0	19	0
13163	Ome, Tokyo	6 Nov, 2013	0	3 [‡]	0
13164	Ome, Tokyo	6 Nov, 2013	0	6 [‡]	0
15322	Tama, Tokyo	16 Dec, 2015	33	3	0
15323	Tama, Tokyo	16 Dec, 2015	108	4 [‡]	0
15324	Tama, Tokyo	16 Dec, 2015	13	6	0

[†] A teneral aptera. [‡] First-instar nymph(s) only.

Many slide-mounted specimens were examined under a light microscope. Since there seemed to be large variation in the sizes of their fore- and mid-legs, all or about 120–180 sub-sampled first-instar nymphs to be alates from the 23 woolly colonies listed in Table 1 were slide-mounted, and one of their fore-femorotrochanters was measured. For the first-instar nymphs in one colony (Exp#6 in Table 1), the length and width of one fore-femorotrochanter (defined in Figure 4(a)) were measured. Measurements were made using a digital camera (FX630; Olympus, Tokyo, Japan) equipped with image analysis software (FlvFs; Flovel, Tachikawa, Japan). It was also recorded whether these first-instar nymphs had the next instar cuticle developing inside (i.e., whether they were in the pharate stage). The percentage of nymphs in the pharate stage roughly corresponds to the degree of their sterility; if the defensive nymphs were completely sterile and did never molt, no nymphs with the next instar cuticle would be found. All non-first-instar nymphs to be alates in two colonies (Exp#1 and 14160 in Table 1) were also slide-mounted to know the composition of instars and whether they were in the pharate stage.

2.4. Defensive Test. To confirm whether aphid nymphs with enlarged legs would really attack other insects, the following experiment was carried out. A total of seven leaves with woolly colonies, which seemed to have produced defensive nymphs, were carefully removed from trees of *Quercus glauca*

in Tama on 28 and 30 September 2015. They were each kept in a plastic container with a sheet of paper, and on the day of collection, two lepidopteran larvae (collected from leaves of *Broussonetia kazinoki*; ca. 3–8 mm) were placed on each colony. When an introduced larva was attacked by aphids within a short period, the larva was placed on a sheet of paper and the attacking behavior was recorded by a video camera attached to a dissecting microscope. After taking a video, the larva and aphid nymphs clinging to it were deposited in a vial of 80% ethanol. When introduced larvae were not attacked by aphids, they were left in the container under a room temperature for approximately 24 hours and examined and deposited in 80% ethanol together with aphids clinging to them. Nine out of 11 larvae attacked by defensive nymphs were macerated in 10% KOH solution, stained with acid fuchsine, and mounted on a glass slide together with the nymphs to examine whether the nymphs really pierced the larval skins under a light microscope. Aphid nymphs that attacked the remaining two larvae were slide-mounted after being detached from the larvae. The remaining aphids were preserved in vials of 80% ethanol, and later defensive first-instar nymphs and other morphs were sorted and counted under a dissecting microscope as mentioned in Section 2.2.

2.5. Transfer Experiment. To confirm whether alates of *Thoracaphis kashifolia* are secondary migrants (alate virginoparae), a transfer experiment was carried out with a test

tree (ca. 2.5 m tall, 5 cm in diameter at 50 cm height) of *Quercus glauca* planted in a garden in Tama. The tree was free from *T. kashifolia*. Eight colonies of *T. kashifolia* were collected from other trees of *Q. glauca* in Tama and Hachioji on 13 October 2015 and kept in plastic containers. Many alates emerged from these colonies. Five twigs of the test tree were chosen and covered with a nylon bag (ca. 50 × 90 cm), and a total of 570 alates were put into the five bags on 14 and 18 October. All leaves in the five bags were examined on 24 October 2015, 22 November 2015, and 7 February 2016.

2.6. Data Analysis. The comparison of fore-femorotrochanteric length between attacking and non-attacking first-instar nymphs was performed by *t*-test, after checking the data for normal distribution (Kolmogorov–Smirnov test) and homogeneity of variance (*F*-test). Differences in the molting rate between the first instar and the remaining three instars were analyzed using Fisher's exact test with Holm's correction for multiple comparisons. Differences in the molting rate between first-instar nymphs with longer forelegs and those with shorter forelegs within three colonies (15226, 15228, and 15230) were analyzed by median test with Fisher's exact test. For the relationships between the fixation date of colonies (collected in Tama in 2015) and the proportion of molting first-instar nymphs, we used a generalized linear model with binomial errors. All statistical analyses were performed with the software R v3.2.3 [33].

3. Results

3.1. Occurrence of Defensive Nymphs. Colony size and composition of our samples are listed in Tables 1 and 2. Ordinary colonies, or colonies that were not covered with long wax filaments, contained only one kind of first-instar nymphs which were small and had ordinary fore- and mid-legs and short marginal setae on the abdomen (Figure 2(b)). Such colonies were seen throughout the year (Table 2), and there is no doubt that these first-instar nymphs would grow to sessile apterae (Figures 1(c) and 2(a)). Colonies covered with long wax filaments, or woolly colonies (Figures 1(a) and 1(b)), were seen from September to early November. In these colonies (except those collected late in the season) there were first-instar nymphs of another kind: they were larger than first-instar nymphs to be apterae and had enlarged fore- and mid-legs with large, strongly curved claws, and long marginal setae on the abdomen (Figure 3(a)). Our analysis of colony composition revealed that these first-instar nymphs would grow to alates through wing-padded nymphal stages and that the first- to fourth-instar nymphs to be alates excreted long wax filaments that made the entire colony like candy floss (Figure 1(b)). Defensive nymphs were first-instar nymphs to be alates (see below).

3.2. Defensive Behavior. In all seven colonies onto which two lepidopteran larvae were introduced, one or both larvae were attacked by defensive nymphs (Figure 1(d), Table 3). Three of the 14 larvae were found hidden under the paper sheet and seemed to have escaped from being attacked by aphid nymphs, while the remaining eleven were attacked by one to

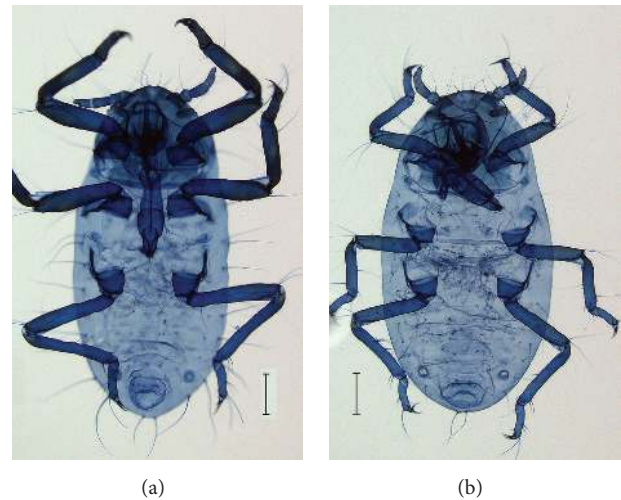


FIGURE 3: First-instar nymphs of the alate generation of *Thoracaphis kashifolia*: (a) typical defensive nymph with enlarged fore- and mid-legs (Tama, Tokyo; 29 September 2015); (b) first-instar nymph produced early in autumn (Tama, Tokyo; 4 September 2015). Scale bars: 100 μ m.

60 nymphs (115 in total). Four nymphs were detached from the larva after being deposited in ethanol, but the remaining 111 were still tightly clinging to the larva. All 115 nymphs were first-instar nymphs to be alates and in the nonpharate stage. It was observed under a dissecting microscope that defensive nymphs were tightly clinging to the lepidopteran larvae with their enlarged mid- and forelegs and seemed to pierce them with their stylets. When attacked, the larvae twisted their bodies and bit off parts of some nymphs clinging to the larvae with their mouthparts (supplementary video 1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/4036571>). Examination of the slide-mounted specimens of nine lepidopteran larvae attacked by aphid nymphs revealed that the stylets of 11 attacking nymphs were inserted in the bodies of the larvae (Figure 5). The maximum length of the stylets inserted in the larvae was 170 μ m; it was approximately as long as a fore-femorotrochanter of a small defensive nymph. The seven colonies also contained 17–76 (mean 39.3) first-instar nymphs to be apterae, but none of them joined to attack the lepidopteran larvae.

3.3. Size and Molting Rate of Defensive Nymphs. There was a large variation in the size of fore- and mid-legs of first-instar nymphs to be alates. Some had distinctly enlarged fore- and mid-legs (Figures 3(a), 4(a), and 4(b)) like *Colophina* soldiers, while some had only slightly enlarged or nearly ordinary legs (Figures 3(b) and 4(c)) and others were intermediate between the two. The length and width of one fore-femorotrochanter are shown as a scattered diagram for almost all first-instar nymphs in a colony (Exp#6) used in the defensive test: 31 nymphs to be apterae and 186 nymphs to be alates including 62 that actually attacked the introduced moth larvae (Figure 6). The 62 nymphs that attacked the larvae had forelegs of various sizes (Figure 6) and their fore-femorotrochanters

TABLE 3: Results of introducing lepidopteran larvae onto seven colonies.

Colony code	Lepidopteran larva: code	Lepidopteran larva: length in mm	Date and time of introduction	Date and time of confirmation	Number of aphids that attacked the larva*
Exp#1	1a	5	28 Sep., 16:00	29 Sep., 15:00	2
Exp#1	1b	4	28 Sep., 16:00	29 Sep., 15:00	0
Exp#2	2a	5	28 Sep., 16:00	28 Sep., 16:05	1
Exp#2	2b	5	28 Sep., 16:00	29 Sep., 16:00	9
Exp#3	3a	4	28 Sep., 16:00	28 Sep., 17:30	8
Exp#3	3b	6	28 Sep., 16:00	29 Sep., 15:00	5
Exp#4	4a	4	30 Sep., 15:50	1 Oct., 15:00	2
Exp#4	4b	3	30 Sep., 15:50	1 Oct., 15:00	5 (1)
Exp#5	5a	5	30 Sep., 15:50	1 Oct., 15:00	0
Exp#5	5b	6	30 Sep., 15:50	1 Oct., 15:00	19
Exp#6	6a	7	30 Sep., 15:50	30 Sep., 16:50	60 (3)
Exp#6	6b	?	30 Sep., 15:50	1 Oct., 15:00	2
Exp#7	7a	3	30 Sep., 15:50	1 Oct., 18:00	2
Exp#7	7b	8	30 Sep., 15:50	1 Oct., 18:00	0

*Number of aphids that were detached from the larva after being deposited in alcohol is in parenthesis.

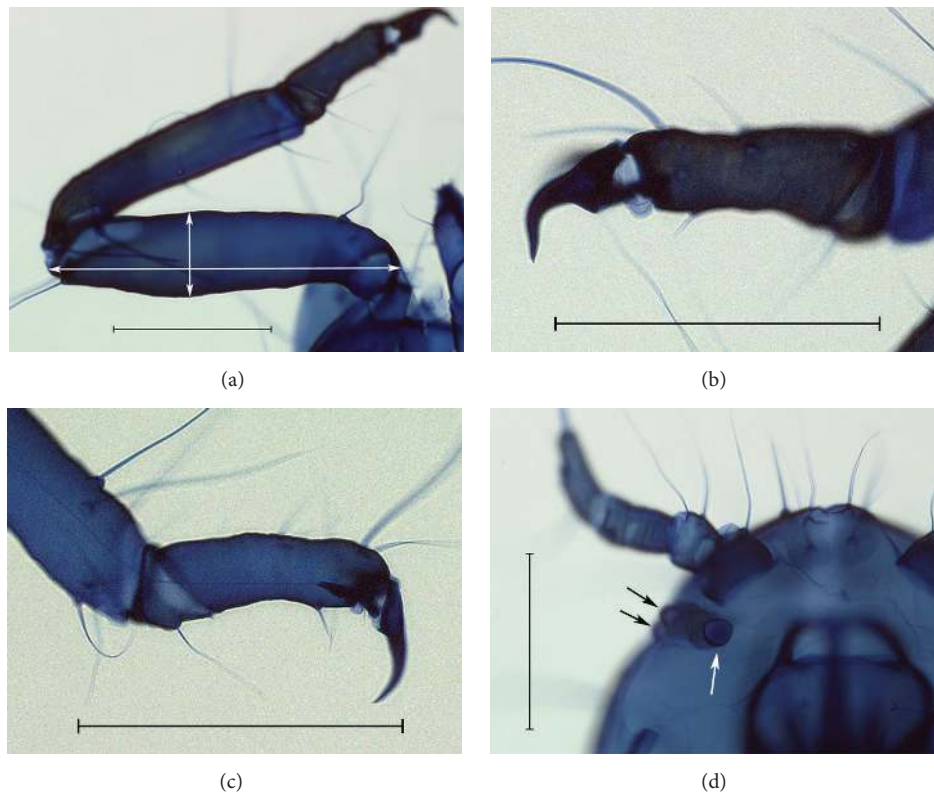


FIGURE 4: Forelegs and head of first-instar nymphs of the alate generation: (a) an entire foreleg of a typical defensive nymph, with double-headed white arrows indicating the length and width of a femorotrochanter defined in this paper; (b) fore tarsus of a typical defensive nymph with thick, strongly curved claws; (c) fore tarsus of a nymph produced early in autumn with slender claws; (d) head of a typical defensive nymph (ventral focus) indicating the downward-directed facet (by a white arrow) and the positions of the remaining two facets (by black arrows) of the left triommatidium. Scale bars: 100 μ m.

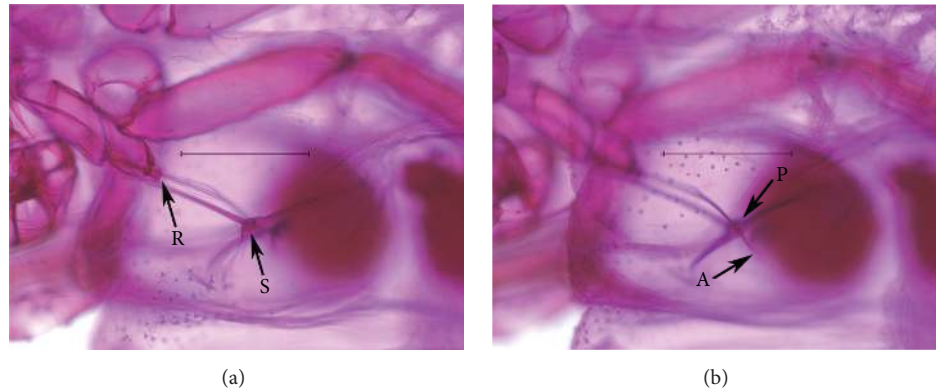


FIGURE 5: Stylets of a defensive nymph stuck through the epidermis of a lepidopteran larva: (a) upper focus; (b) lower focus. Capital letters on the figure indicate the apex of the rostrum of the attacking aphid (R), the socket of a seta on the epidermis of the lepidopteran larva (S), the apices of the aphid's stylets (A), and the presumed puncture point (P). The stylets are extended from the apex of the rostrum (R) to the point A through the point P below the socket (S). Scale bars: 100 μm .

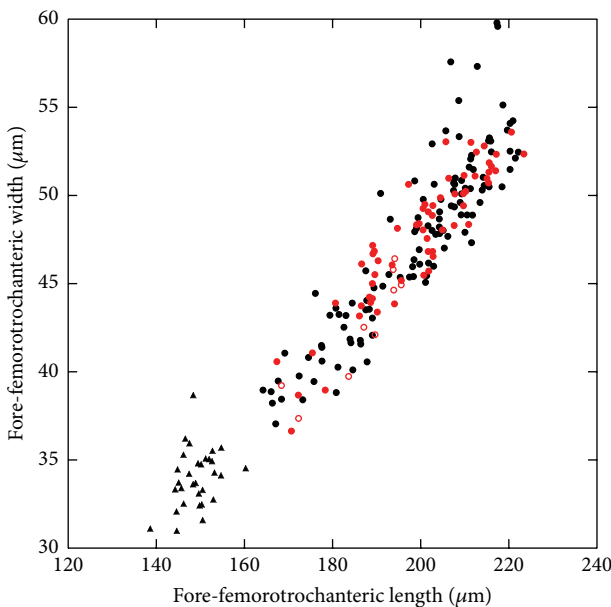


FIGURE 6: Scattered diagram of the length and width of one fore-femorotrochanter for 31 first-instar nymphs to be apterae (triangle) and 186 first-instar nymphs to be alates (circle) including 62 that actually attacked the introduced moth larvae (red closed circle) in a colony (Exp#6). First-instar nymphs (to be alates) that had the second-instar cuticle developing inside are indicated by red open circles.

were not longer than those of the remaining 124 nymphs that did not join the attack (t -test, $p = 0.418$).

First-instar nymphs to be alates in three colonies sampled early in the season (on 4, 12, and 20 September) had forelegs that were shorter than those sampled later in the season (Figure 7; see also Figure 8), and their “molting rates” (sensu Akimoto [11]), or the proportions of those nymphs that had the second-instar cuticle developing inside, were high, 27.5, 16.8, and 9.1%, respectively (Table 1). On the other hand, first-instar nymphs in the seven experimental colonies

(colonies Exp#1–7) collected near the end of September and the colonies collected thereafter showed small values of the molting rate, less than 5%, and some (e.g., colonies 14158, 14160, and 15258) even zero percent (Table 1). Picking up 16 colonies collected in Tama in 2015 from Table 1, the molting rate of first-instar nymphs decreased with the date of fixation (generalized linear model with binomial errors, $\chi^2 = 117.5$, $p < 0.0001$). The molting rate of first-instar nymphs in colonies sampled near the end of September and thereafter, colonies Exp#1 and 14160, for example, was also lower when compared with the second- to the fourth-instar nymphs (Figure 9). (Note that in colony Exp#1, alate adults had not yet appeared. Because the colony was relatively young, the molting rate of the fourth-instar nymphs was low.)

In the early-sampled three colonies (for colony 15228, see Figure 8(a), left), first-instar nymphs with shorter forelegs tended to have the next instar cuticle developing inside (median test with Fisher's exact test; $p < 0.01$). In colonies sampled later in the season, the molting rate was so low that the tendency could not be confirmed statistically; however, defensive nymphs with large forelegs molt at least at times. In colony 15257 (sampled on 13 October 2015), two out of 46 first-instar nymphs to be alates had the next-instar cuticle; their fore-femorotrochanters were 217 and 216 μm long, the fifth and sixth longest in that colony.

3.4. Results of the Transfer Experiment. Offspring of the alates introduced into the five bags colonized on a few leaves in four bags. When examined on 22 November 2015, a total of 22 aphids (2 apterous adults and 20 nymphs to be apterae) were seen on the upper sides of seven leaves of *Quercus glauca*. On 7 February 2016, 15 apterous adults and one (presumably dead) nymph were found on four leaves. Although, taking the number of the mother alates (more than 500) into consideration, the success rate in colonization may be low, the result shows that the alates are not sexuparae but secondary migrants. All first-instar nymphs born in the glass vials were to be apterae, or of the same phenotype as in Figure 2(b). The ten alates (from colony 15241) collected in Kyoto and the ten

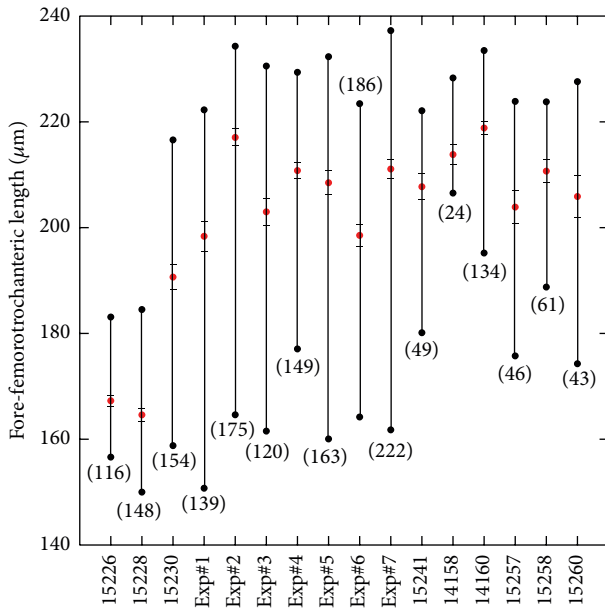


FIGURE 7: Lengths of fore-femoro-trochanters of first-instar nymphs to be alates for 16 woolly colonies. Mean \pm 2SE are shown by a red closed circle and two short horizontal bars on the vertical line which indicates the range. Number in parenthesis indicates the sample size. The colony codes shown below the horizontal axis are the same as in Table 1.

alates (from colony UE1013) collected in Tsuchiura also turned out to be secondary migrants. No sexupara was found in this study.

3.5. Predators Found in the Field. Colonies of *Thoracaphis kashifolia* were rather free of predators. From the 23 sampled woolly colonies and the 22 ordinary colonies we found no predator but a few dead syrphid eggs; it is not certain whether they were killed by defensive nymphs. Only one predator we found from woolly colonies was a hemerobiid larva (ca. 9 mm long). When found (in Tama on 14 October 2015), it was still alive but three defensive nymphs were tightly clinging to near the tip of its abdomen and one clinging to its thorax, near the base of its left mid-leg. We also found a chrysoptid larva (ca. 10 mm long) from an ordinary colony, with a dead apterous adult of *T. kashifolia* on its back (in Tama on 19 August 2015). No ants were seen attending woolly or ordinary colonies.

4. Discussion

4.1. Convergent and Peculiar Features of the Defensive Nymph. As described in Section 3.2, first-instar defensive nymphs of *Thoracaphis kashifolia* clasp a predator with their enlarged fore- and mid-legs and pierce it with their stylets. Such attacking behavior is also known in soldiers of the eriosomatine *Colophina clematis* [1]. Their thickened legs with large, strongly curved claws (Figures 4(a) and 4(b)) are similar to those of the soldiers of *Colophina* spp. in shape (cf. [34, 35]), which is no doubt due to convergent evolution. On the other hand, the defensive nymphs of *T. kashifolia* have

an idiosyncratic attacking device. The aphids of the tribe Nipponaphidini on the secondary host have long stylets, which are far longer than the rostrum [36], like adelgids [37]. When not extended, the stylets are kept coiled in the head; it is not easy to measure how long they are. In one slide-mounted defensive nymph, the stylets were extended about 330 μm from the apex of the rostrum. Defensive nymphs often inserted their stylets deep in the body of a lepidopteran larva to which they were clinging; the length of the stylets inserted in the larva was up to 170 μm (Section 3.2). Such deep insertion of stylets has been unknown from defensive nymphs of other groups. Defensive nymphs of *T. kashifolia* may use the stylets inserted in the body of an enemy as an anchor, so as not to be detached easily from it.

Another peculiar feature of the defensive nymph of *T. kashifolia* is its eyes. An aphid nymph of Hormaphidinae has a pair of triommatidia, each of which consists of three facets. In the first-instar nymphs of *T. kashifolia* (both nymphs to be apterae and to be alates), one facet is located apart from the remaining two, on the underside of the head and directed downward (Figure 4(d)). The same type of triommatidia is known in the first-instar nymphs of *Metathoracaphis isensis* [31], which also forms colonies on the upper sides of leaves of the host oak (*Quercus gilva*). This suggests that such triommatidia may be an adaptation in the life on the upper side of a leaf and that the downward-directed facets might enable the nymphs living on the upper side to perceive enemies on the underside through the leaf tissue.

4.2. Soldier-Caste Differentiation? Two morphologically distinct phenotypes of first-instar nymphs occur in *Thoracaphis kashifolia*: first-instar nymphs to be apterae (Figure 2(b)) and those to be alates (Figure 3). The former are clearly smaller than the latter as exemplified in Figure 6. Such dimorphism also occurs in the nipponaphidine *Neothoracaphis quercicola* [38], *N. yanonis* and *N. saramaensis* (our unpublished results), and *Reticulaphis* sp. [39], all of which produce tiny, flattened apterae on leaves and may have evolved in association with the miniaturization and/or flattening of their aleyrodiform apterae. Coexistence of two morphologically distinct generations in a colony where first-instar nymphs of one generation play a defensive role is already known in the gall-forming aphid *Pemphigus spyrothecae* [40, 41]. The issue we address below is whether caste differentiation occurs within the first-instar nymphs of the alate generation, and not between the apterous and alate generations.

As mentioned in Section 3.3, first-instar nymphs produced early in September had smaller fore- and mid-legs than those produced later. Also, their molting rate was higher than the latter. These facts indicate that a kind of soldier-caste differentiation occurs in the alate generation of *T. kashifolia*; first-instar nymphs as shown in Figure 3(a) are soldiers and those in Figure 3(b) are to be reproductives. However, there were many first-instar nymphs with forelegs of intermediate sizes (Figure 7), and nymphs with forelegs of various sizes attacked lepidopteran larvae (Figure 6). In addition, although the molting rate of first-instar nymphs produced late in the season was extremely low (from less than 5% to zero), some still tried to molt to the next instar. The demarcation

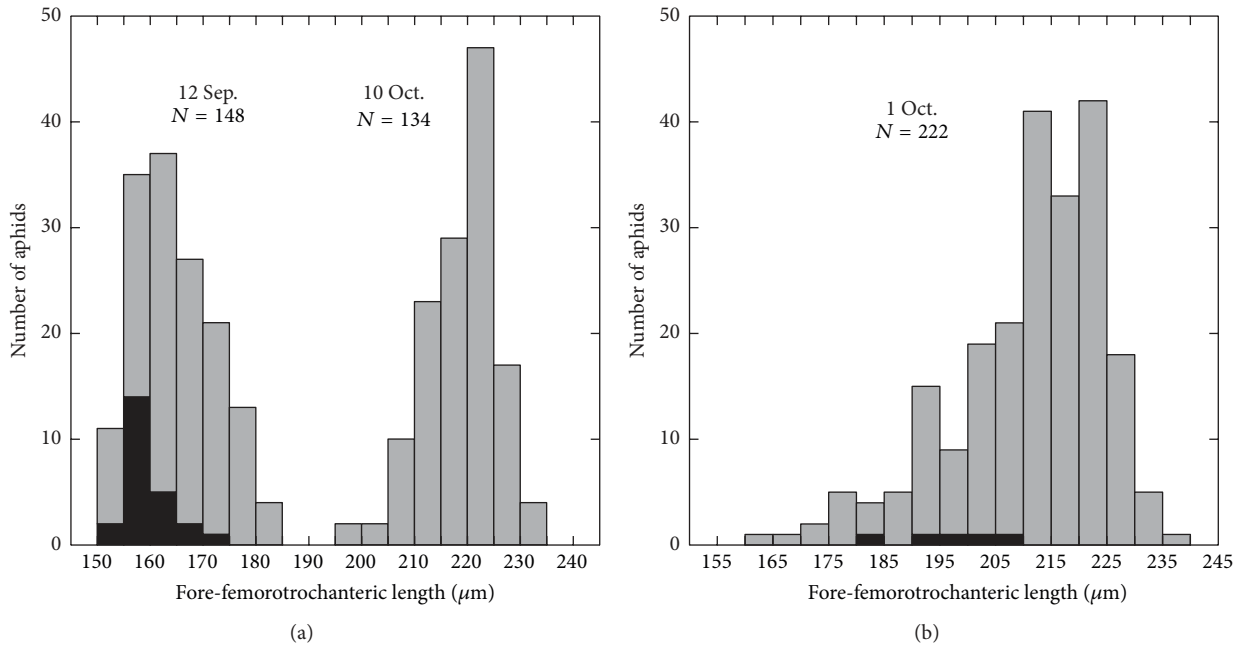


FIGURE 8: Frequency distribution of the fore-femoro-trochanteric length of first-instar nymphs to be alates with (indicated by black) or without (indicated by grey) the next-instar cuticle developing inside. (a) Colony 15228 (left) on 12 September 2015 and colony 14160 (right) on 10 October 2014; (b) colony Exp#7 on 1 October 2015.

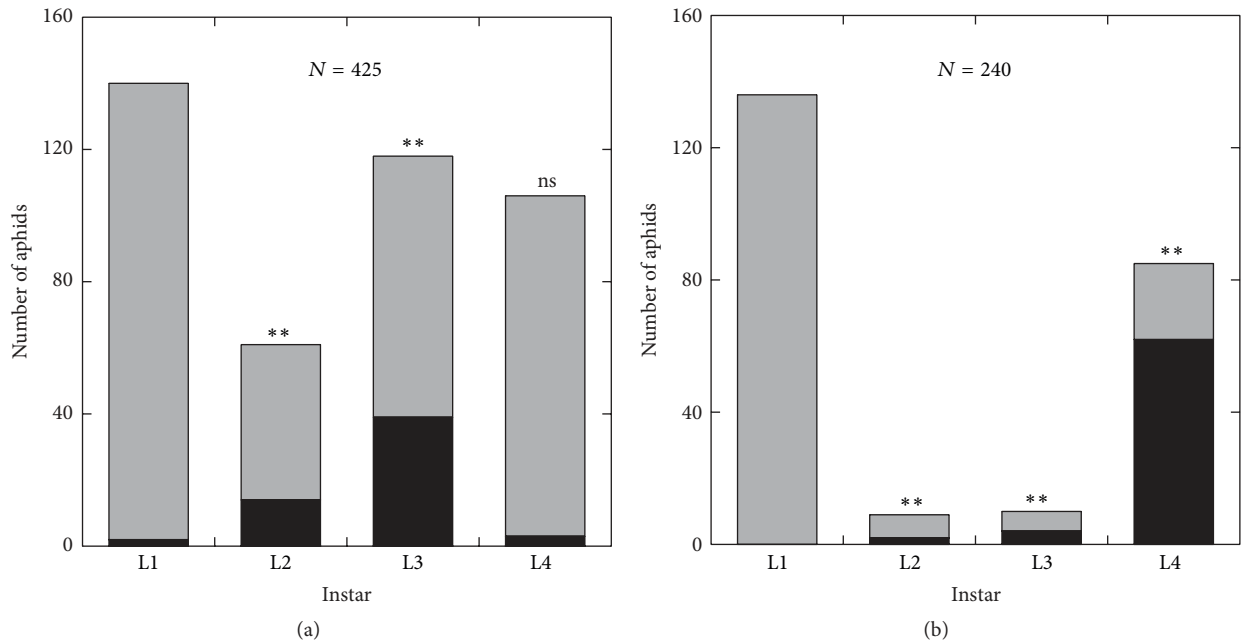


FIGURE 9: Number of the first- to the fourth-instar nymphs with (indicated by black) or without (indicated by grey) the next-instar cuticle developing inside. (a) Colony Exp#1 (29 September 2015); (b) colony 14160 (10 October 2014). L1, L2, L3, and L4 denote the first-, second-, third-, and fourth-instar nymphs, respectively. Significant differences ($p < 0.01$) in the molting rate between the first instar and the remaining three instars are indicated by double asterisks above each column (Fisher’s exact test with Holm’s correction).

between the two castes is therefore not clear cut. This is an incipient kind of soldier differentiation, or colonies of *T. kashifolia* are at an intermediate state between those with sterile soldiers (as in *Colophina clematis* [1]) and those with “monomorphic” defensive nymphs which all are supposed to

have the potential to grow into reproductives (as in *Hemipodaphis persimilis* [11] or *Hamamelistes* spp. [13, 42]).

4.3. Life Cycle of *Thoracaphis kashifolia* in Japan. The present study confirmed that the life cycle of *Thoracaphis kashifolia*

is anholocyclic in Japan, as had been suggested by Takahashi [27]. That is, aleyrodiform apterous adults propagate themselves by parthenogenesis throughout the year on leaves of the oak *Quercus glauca*. They give birth to first-instar nymphs that grow to alates from early September. These alates are secondary migrants; they migrate to trees of *Quercus glauca*. First-instar nymphs of the alate generation play a defensive role. During October, the number of the alate generation decreases steeply. The seven colonies used in the defensive test (fixed on 29 September and 1 October) contained 425–1203 (mean 632) nymphs to be alates, while six colonies sampled on 26 and 27 October contained only 25–85 (mean 64) such nymphs (Table 1); the number of nymphs decreased to approximately 1/10 during 25–28 days. The number of first-instar nymphs to be alates decreased even more steeply, to 6/1000. This steep decrease could not be explained by molting of the first-instar nymphs, because the molting rate during this period was very low (Table 1, Figure 9). It was no doubt due to the high mortality of the defensive nymphs in exposed colonies.

We still do not know whether or how often a new colony of *T. kashifolia* begins with a single aphid. Our preliminary (unpublished) observations showed that some new colonies were formed on leaves near a woolly colony by more than one first-instar nymph to be aptera (e.g., colonies 15236–9 in Table 2). Although new colonies will also be formed by alates (Section 3.4), we have not yet confirmed this type of colony founding under natural conditions.

5. Conclusion

In this paper we made it clear that an incipient soldier-caste differentiation occurs in the alate generation of the aphid *Thoracaphis kashifolia*. This is the first discovery of defensive individuals from Nipponaphidini on their secondary hosts. The discovery was a little surprise because apterous adults of this group are sessile and seem to be protected by the hard exoskeleton. The defensive individuals are first-instar nymphs of the alate generation and therefore are produced only when aphids of the alate generation are present.

Competing Interests

The authors have no affiliations or involvement with any organization that has a financial interest in the results discussed in this paper.

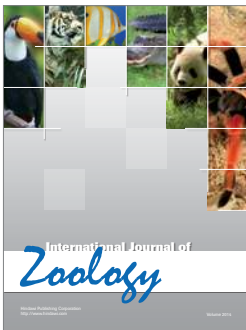
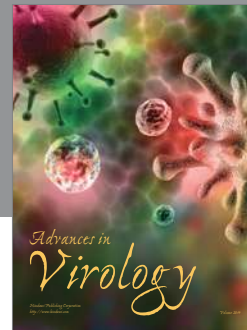
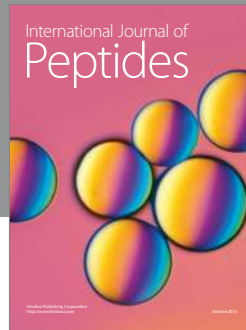
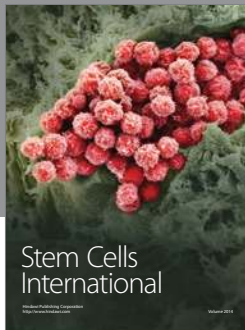
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