Evolutionary Ecology Research, 2004, **6**: 695–707

Impact of differential parasitoid attack on the number of chambers in multilocular galls of two closely related gall midges (Diptera: Cecidomyiidae)

K. Tabuchi* and H. Amano

Laboratory of Applied Entomology and Zoology, Faculty of Horticulture,
Chiba University, Matsudo, Chiba 271-8510, Japan

ABSTRACT

We examined the influence of differential selection by natural enemies on the number of chambers in the galls of two closely related gall midges: *Asteralobia sasakii* and *A. soyogo*. To evaluate the selection pressure imposed by parasitoid wasps, we investigated the number of larval chambers (as represented by the number of larvae) and the rate of parasitism in each gall. Galls with smaller numbers of either species of cecidomyiid larvae were attacked more frequently by ectoparasitoids. In *A. sasakii* galls, the overall rate of attack by ectoparasitoids was higher than in *A. soyogo* galls, especially in galls with many larvae. Midge larvae of both species that hatched from larger egg clutches were attacked more frequently by endoparasitoids. In *A. sasakii*, the overall rate of attack by endoparasitoids was lower than in *A. soyogo*, especially in large clusters of larvae. Consequently, the final survival rate of *A. sasakii* peaked at higher larval numbers (14.5 per gall in 2000–01 and 10.1 per gall in 2001–02) than for *A. soyogo* (5.5 per gall in 2000–01 and 6.4 per gall in 2001–02). The mean number of larvae per gall was also higher in *A. sasakii* than in *A. soyogo*. Differences in the selection pressures exerted by natural enemies led to differences in the numbers of chambers in galls of the two different midge species and, in turn, different oviposition behaviours of midge females, reflecting variations in counter-strategies against parasitoids.

Keywords: Aquifoliaceae, *Asteralobia*, clutch size, gall midge, *Ilex*, parasitoid.

INTRODUCTION

Among herbivores, predation by natural enemies is one of the most important selective agents (e.g. Krebs, 2001). Selection pressure imposed by natural enemies can cause both biological and evolutionary responses in herbivores. Because immature forms of endophytic insect herbivores (including gall-makers) spend all their larval stages at a single site on a certain host plant, they cannot avoid predation by moving to other, less dangerous sites. Therefore, many strategies for avoiding predation, such as physiological defence, temporal

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* Author to whom all correspondence should be addressed. e-mail: tabuchik@graduate.chiba-u.jp
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avoidance and spatial avoidance, have evolved in these insect species (e.g. Gross, 1993; Hoffmeister *et al.*, 1999).

Galls are not only a form of plant tissue, but they also can be considered as an extended phenotype of the gall-forming insect (Dawkins, 1982; Stone and Schönrogge, 2003). Biological and evolutionary responses of gall-making insects towards parasitoids and predators may alter the morphology of the gall, including its size, shape and structure (Cornell, 1983; Weis and Abrahamson, 1986; Price *et al.*, 1987; Zwölfer and Arnold-Rinehart, 1994; Stone and Cook, 1998). In particular, gall size can play an important part in providing a refuge for gall-making insects from parasitoids and predators (Weis, 1985; Price and Clancy, 1986; Price, 1988; Zwölfer and Arnold-Rinehart, 1994; Freese and Zwölfer, 1996; but see also Rossi *et al.*, 1992). Gall size differences may be important in insects that cause unilocular galls, because the gall acts as a physical wall for the natural enemies of the larvae: a large, thick wall protects the insect better (Weis, 1985; Price and Clancy, 1986; van Hezewijk and Roland, 2003). In insects that cause multilocular galls, the success of attacks by natural enemies may depend on the number of larvae inhabiting each gall, in addition to gall size itself (Jones, 1983; Weis *et al.*, 1983; Zwölfer and Arnold-Rinehart, 1994; Freese and Zwölfer, 1996). Therefore, selection pressure from natural enemies may affect the oviposition habits (i.e. clutch size) of adult females (Weis *et al.*, 1983).

Previous studies of the effects of selection pressure on the number of larvae in each gall have targeted only one species of galling insect on one host plant in one location (Jones, 1983; Weis *et al.*, 1983). For example, selection differentials with regard to the size of the gall refuge have been examined in 16 populations of the goldenrod gall fly *Eurosta solidaginis*, which forms unilocular galls, over five generations (Weis *et al.*, 1992; Weis and Kapelinski, 1994; Weis, 1996). Comparative studies are useful for gaining an overall picture of the importance of clutch size in the biological and evolutionary response of oviposition habits to attack by natural enemies.

We tested the hypothesis that differential attacks by parasitoids place different selection pressures on the number of larvae forming each gall. We observed two generations of two closely related gall midges (Diptera: Cecidomyiidae), *Asteralobia sasakii* on *Ilex crenata* and *A. soyogo* on *I. integra*. To evaluate the responses of the midges to parasitoids, we examined the following three features: first, we observed the number of chambers (= initial number of larvae) in each gall for each midge; second, we investigated the rate of parasitism in larvae sharing galls; and third, we observed the behaviour of each parasitoid species in attacking each midge species.

Natural history of the system

Asteralobia sasakii and *A. soyogo* are widely distributed in Japan (Yukawa and Masuda, 1996; Tokuda *et al.*, 2002). Both midges form multilocular galls on the axillary buds of several species of *Ilex* tree (Aquifoliaceae) (Monzen, 1937; Yukawa and Masuda, 1996; Tokuda *et al.*, 2002). The distributions of these midges overlap in many parts of Japan, but they segregate by using different host plants (Tokuda *et al.*, 2002).

The life histories of *A. sasakii and A. soyogo* inhabiting *I. crenata* and *I. integra*, respectively, are described in Tabuchi and Amano (2003a). (These authors refer to *A. soyogo* as an *A. sasakii* population on *I. integra*.) All individuals of *A. sasakii* and most individuals of *A. soyogo* have a univoltine life cycle: adults emerge in late April to June after overwintering in galls on the shoots. After mating, adult females oviposit into the axillary buds of shoots. It is unknown whether single galls are produced by single females or by several females. The galls mature by autumn, and the larvae complete their growth in October. The mature larvae overwinter in the galls and then pupate in the following March. Some individuals of *A. soyogo* have 2- or 3-year life cycles.

Asteralobia soyogo on *I. integra* was misidentified as *A. sasakii* until recently (Yukawa, 1971; Yukawa and Masuda, 1996), because both midges have similar life histories, galling positions on plants and gall shapes. However, allochronic reproductive isolation has been reported between *A. sasakii* on *I. crenata* and *A. soyogo* on *I. integra*, with different adult emergence periods (Tabuchi and Amano, 2003a,b), as well as differences in morphological characteristics (M. Tokuda and J. Yukawa, personal communication).

During their larval periods, *A. sasakii* and *A. soyogo* are attacked by four and three parasitoid wasps, respectively (Tabuchi and Amano, 2003a). *Inostemma* sp. (Platygastridae, endoparasitoid) and *Torymus* sp. (Torymidae, ectoparasitoid) wasps attack only *A. sasakii*, whereas *Gastrancistrus* sp. (Pteromalidae, endoparasitoid) attacks only *A. soyogo*. Two ectoparasitic wasps, *Mesopolobus* sp. (Pteromalidae) and *Bracon tamabae* (Braconidae), attack both gall midges. Much of the mortality of larvae and pupae of *A. sasakii* and *A. soyogo* is caused by the parasitoids: unknown lepidopteran larvae occasionally feed on galls of both midges and act as supplementary factors in mortality (K. Tabuchi and H. Amano, unpublished).

MATERIALS AND METHODS

Field studies were conducted at the Matsudo campus of Chiba University (in the city of Matsudo in Chiba Prefecture) near Tokyo. The altitude of the campus is about 26 m above sea level (35°47′N, 139°54′W).

The fate of midge larvae

In 2000–01 and 2001–02, mature galls of *A. sasakii* on *I. crenata* and *A. soyogo* on *I. integra* were randomly collected in the field (Table 1). In the laboratory, we measured the diameters of the galls collected and then dissected them to count the number of larvae in each gall under a binocular microscope. Immature galls containing dead larvae before chamber formation and withered galls were excluded from measurements of gall size. When we dissected the galls, we also observed the incidence of parasitism of each larva. The gall samples were taken between November and May in both years. At this time of the year, the galls and larvae are fully mature, and most parasitoid attacks have ceased (Tabuchi and

Table 1. The total number of galls and larvae of *A. sasakii* and *A. soyogo* used in the survey

	Year	No. of trees	No. of galls	No. of larvae
A. sasakii on *I. crenata*	2000–2001	2	251	994
	2001–2002	1	197	1006
A. soyogo on *I. integra*	2000–2001	1	252	728
	2001–2002	1	194	518

Amano, 2003a). The survey was conducted at weekly intervals, and most samplings were conducted in April and May for *A. sasakii* and in March and April for *A. soyogo*, because of the life history of each midge species (Tabuchi and Amano, 2003a). In each sampling, 6–49 galls were collected from the census tree. Each year, one tree of each species was selected for gall samplings, except that two trees of *I. crenata* were sampled in 2000–01 (Table 1). Different trees were used for samplings in different years to avoid any influence of the previous year's survey.

Ectoparasitoid attacks

Both endo- and ectoparasitoids can be found as larval stages in the gall (November to April; Tabuchi and Amano, 2003a). Identification of the larval stages of the ectoparasitoids is not possible. Therefore, for both midge species we randomly clipped five shoots with galls from several census trees between March and October 2002 at 7- to 20-day intervals. The collected galls were dissected under a binocular microscope, and the larvae and pupae of the parasitoids inside were reared in gelatin capsules at 25 ± 2°C in the laboratory and identified when they reached adulthood.

Data analysis

All analyses were conducted with JMP statistical software (SAS Institute Inc., 2000). For each midge species, simple linear regression was used to analyse the relationship between number of midge larvae per gall and gall diameter, and an analysis of covariance (ANCOVA) slope homogeneity test was used to test parallelism between regression lines. When the regression lines were parallel, ANCOVA's basic design was used to determine whether the y-intercepts were significantly different. We used a t-test to compare the number of larvae per gall between *A. sasakii* and *A. soyogo*.

For each midge species, simple logistic regression with binomial errors was used to analyse the relationship between number of larvae per gall and rate of combined parasitism by both ecto- and endoparasitoids in 2000–01 and 2001–02. When larvae of both ecto- and endoparasitoid wasps were observed with a midge larva, we counted this as ectoparasitoid attack, because the larvae of ectoparasitoids eventually feed on both the midge larva and the endoparasitoid wasp. We used a multiple logistic regression model to establish homogeneity between the fitted models. For each species, quadratic logistic regression with binomial errors was also used to analyse the relationships between number of larvae per gall and survivorship of larvae. When the regression line was statistically supported ($P < 0.05$), the optimum number of larvae per gall, which represented the maximum survival rate, was estimated for each midge species. (An estimate of the peak of the function could be obtained from the apex of the quadratic functional equation.)

In the two previously mentioned logistic regression analyses, data were categorized by number of larvae per gall, and data of parasitoid attacks were counted by each category. When only one gall was collected in any category, these galls were excluded from the analysis to avoid bias.

We estimated the extent of the effect of both directional and stabilizing selection on the gall-makers from the number of larvae inhabiting each gall. The action of directional selection on a quantitative character can be measured as s, the selection differential: this is defined as $s = \bar{z}^* - \bar{z}$, where $\bar{z}$ is the mean phenotype before selection and $\bar{z}^*$ is the mean phenotype of the selected individuals (Lande and Arnold, 1983). The stabilizing selection differential, *C*, quantifies the change in phenotypic variance caused by stabilizing selection (Lande and Arnold, 1983), independent of any change caused by directional selection. It is defined as $C = V_{P*} - V_P + s^2$, where V_P is the pre-selection phenotypic variance in a character and V_{P*} is the post-selection variance (see Lande and Arnold, 1983, eqn. 13b).

RESULTS

Difference in numbers of larvae inhabiting galls of the two midges

In both midge species, there were positive relationships between the number of larvae in each gall and gall diameter (Fig. 1). The slopes of the regression lines for *A. sasakii* and *A. soyogo* did not differ (ANCOVA hypothesis of parallel slopes: $F_{1,415} = 0.13$, not significant; ANCOVA basic design: $F_{1,415} = 2.84$, not significant). The number of larvae per gall, however, was significantly different (*t*-test: $P < 0.0001$) between the two midges in each year (Fig. 2): galls of *A. sasakii* contained significantly more larvae than those of *A. soyogo*.

Relationship between rate of parasitism and number of larvae inhabiting galls of the two midges

The relationships between number of larvae per gall and rate of parasitism by ecto- and endoparasitoids are shown in Fig. 3. In both species, smaller galls with fewer larvae generally suffered heavier attacks by ectoparasitoids (Fig. 3A), whereas larger galls with more larvae had higher rates of attack by endoparasitoids: *Inostemma* sp. on *A. sasakii* and *Gastrancistrus* sp. on *A. soyogo* (Fig. 3B).

Heterogeneity of attack by endo- and ectoparasitoids was observed between the two midge species. The fitted models were significantly different between the two species for each generation (Table 2 and Fig. 3). The rate of parasitism of *A. sasakii* by ectoparasitoids was higher than that of *A. soyogo* in the 2000–01 survey (Fig. 3A). In the 2001–02 survey, the fitted curve showed that ectoparasitoids attacked the large *A. sasakii* galls containing many

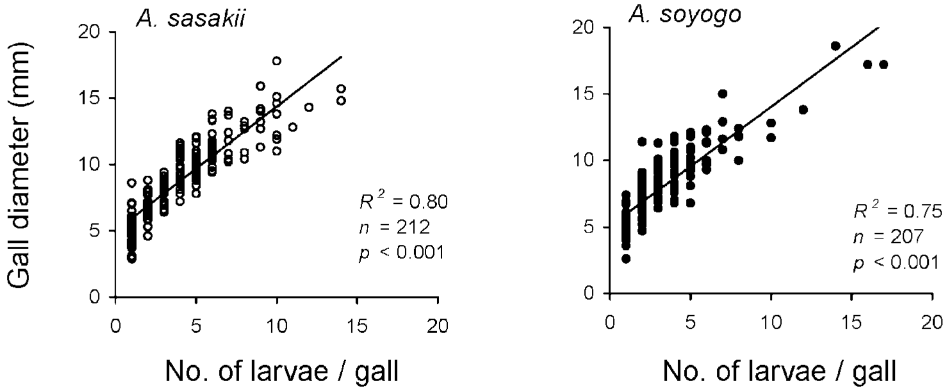

Fig. 1. Relationship between number of larvae per gall and gall diameter in 2000–01. Regression lines: $y = 0.94x + 5.00$ for *A. sasakii* on *I. crenata*, $y = 0.90x + 5.07$ for *A. soyogo* on *I. integra*.

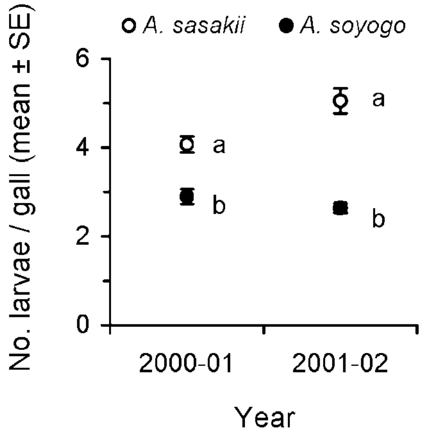

Fig. 2. Comparison of the number of larvae per gall between *A. sasakii* and *A. soyogo* in 2000–01 and 2001–02. Different letters within each year indicate significant difference by *t*-test ($P < 0.0001$).

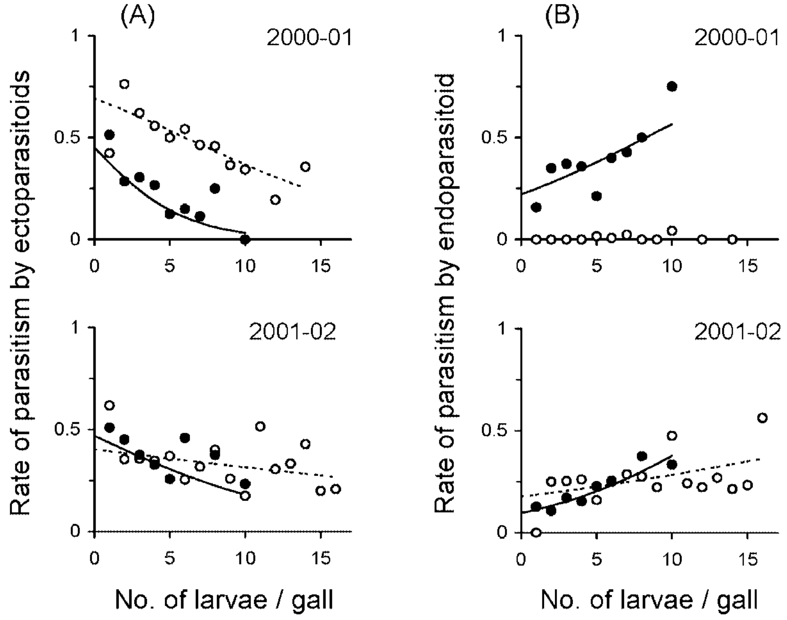

Fig. 3. Relationships between number of larvae per gall and (A) rate of parasitism by ectoparasitoids and (B) rate of parasitism by endoparasitoids for *A. sasakii* (open circles) and *A. soyogo* (solid circles) in 2000–01 and 2001–02. Each point is the mean for rate of parasitism; untransformed data are shown. Dotted lines and solid lines indicate the predictions of logistic regression models for *A. sasakii* and *A. soyogo*, respectively. (A) For *A. sasakii* in 2000–01: $y = 1 - 1/\exp(0.92 - 0.13x)$, $P < 0.0001$; in 2001–02: $y = 1 - 1/\exp(-0.39 - 0.04x)$, $P < 0.05$. For *A. soyogo* in 2000–01: $y = 1 - 1/\exp(-0.19 - 0.32x)$, $P < 0.0001$; in 2001–02: $y = 1 - 1/\exp(-0.12 - 0.14x)$, $P < 0.01$. (B) For *A. sasakii* in 2000–01: $P > 0.05$; in 2001–02: $y = 1 - 1/\exp(-1.53 + 0.06x)$, $P < 0.001$. For *A. soyogo* in 2000–01: $y = 1 - 1/\exp(-1.25 + 0.15x)$, $P < 0.0001$; in 2001–02: $y = 1 - 1/\exp(-2.23 + 0.17x)$, $P < 0.001$.

larvae (more than 5 larvae per gall) more frequently than *A. soyogo* galls (Fig. 3A). In the 2000–01 survey, the rate of parasitism of *A. sasakii* by the endoparasitoid (*Inostemma* sp.) was very low, no matter how big or small the gall was, and the relationship between rate of parasitism and larval density in the gall was not significant (Fig. 3B). In the 2000–01 survey of *A. soyogo* for endoparasitoids (*Gastrancistrus* sp.), the rate of parasitism was clearly higher than that of *A. sasakii* (Fig. 3B). The relationships between rate of parasitism by endoparasitoids and number of larvae per gall were significant in both species in the 2001–02 survey (Table 2), but the fitted models for both species were significantly different from each other: the increase in the rate of parasitism was sharper in *A. soyogo* (Fig. 3B).

For both *A. sasakii* and *A. soyogo*, mortality induced by natural enemies resulted in both directional and stabilizing selection for number of larvae per gall (Table 3). Ectoparasitoid

Table 2. Summary of multiple logistic regression models testing the effect of species and the number of larvae per gall on the rate of parasitism

	Coefficient	Wald's χ^2	*P*
Attack by ectoparasitoids			
2000–01 survey			
Species	0.95	143.25	<0.001
No. of larvae per gall	−0.22	55.61	<0.001
Species × no. of larvae per gall	0.09	9.58	0.002
2001–02 survey			
Species	0.19	4.46	0.035
No. of larvae per gall	−0.09	12.88	<0.001
Species × no. of larvae per gall	0.05	4.16	0.041
Attacks by endoparasitoids			
2001–02 survey			
Species	−0.01	0.01	0.928
No. of larvae per gall	0.12	21.36	<0.001
Species × no. of larvae per gall	−0.06	4.90	0.027

Table 3. Selection differentials by natural enemies on the number of midge larvae inhabiting each gall

		Selection differential			
		Directional selection, *s*			
Species	Year	Total	Ectoparasitoids	Endoparasitoids	Stabilizing selection, *C*
A. sasakii	2000–01	1.16	1.19	−0.03	1.34
	2001–02	−0.20	0.20	−0.55	−1.11
A. soyogo	2000–01	0.30	0.62	−0.11	0.03
	2001–02	0.21	0.38	−0.16	−0.08

attack represented a selection force towards rising numbers of chambers per gall, and endoparasitoid attack represented a selection force towards declining numbers of chambers per gall (Table 3). For *A. sasakii*, the direction of selection pressure differed within the two generations. The rising force overwhelmed the declining force in the 2000–01 survey, whereas in the 2001–02 survey the latter force was slightly greater than the former. In contrast, in *A. soyogo* across the two generations, the selection pressure towards a rise in the number of larvae per gall was generally greater than that towards a decline (Table 3). In addition, changes in phenotypic variance (*C*) varied between the two midges in both years (Table 3).

Survivorship of larvae inhabiting galls with different numbers of larvae

Figure 4 shows the survivorship of both *A. sasakii* and *A. soyogo* larvae in galls with different larval numbers. Peak survival rates were significantly different between the species in both sampling years (Fig. 4). The survivorship of *A. sasakii* was highest in large galls containing many larvae, whereas for *A. soyogo* it was highest in smaller galls containing fewer larvae (Fig. 4). In *A. sasakii*, maximum survivorship ($P_{\max}$) occurred at 14.5 larvae per gall in 2000–01 and 10.1 larvae per gall in 2001–02, whereas in *A. soyogo* it was 5.5 larvae per gall in 2000–01 and 6.4 larvae per gall in 2001–02.

Types of parasitoid fauna in the galls

The contents of the galls of the two midge species are shown in Table 4. For the two ectoparasitoids attacking both *A. sasakii* and *A. soyogo* (*B. tamabae* and *Mesopolobus* sp.), *B. tamabae* numbers were lower than those of *Mesopolobus* sp. The number of *Torymus* sp., which attacks only *A. sasakii*, was close to that of *Mesopolobus* sp. (Table 4).

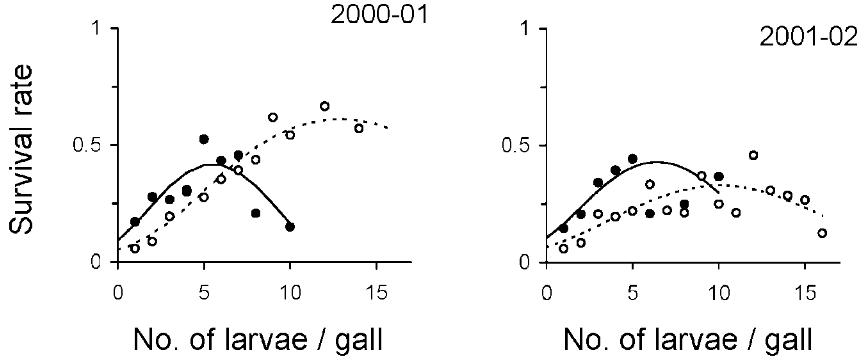

Fig. 4. Relationship between number of larvae per gall and survival rate for *A. sasakii* (open circles) and *A. soyogo* (solid circles) in 2000–01 and 2001–02. Each point indicates the mean for survival rate; untransformed data are shown. Dotted and solid lines indicate the predictions by logistic regression models for *A. sasakii* and *A. soyogo*, respectively. *A. sasakii*: $y = 1 - 1/\exp\,(-2.96 + 0.53x - 0.02x^2)$, $P < 0.001$ in 2000–01; $y = 1 - 1/\exp\,(-2.68 + 0.39x - 0.02x^2)$, $P < 0.05$ in 2001–02. *A. soyogo*: $y = 1 - 1/\exp\,(-2.29 + 0.71x - 0.06x^2)$, $P < 0.001$ in 2000–01; $y = 1 - 1/\exp\,(-2.16 + 0.58x - 0.05x^2)$, $P < 0.01$ in 2001–02.

Table 4. Contents in galls of *A. sasakii* and *A. soyogo* from March to October in 2002

			No. in all galls examined								
				Ectoparasitoids[a]			Endoparasitoids[b]				
	No. of shoots	No. of galls	Total	Bt	Me	To	In	Ga	Unknown parasitoids[c]	No. of midges	Unknown[d]
A. sasakii	177	242	1170	5	51	54	218	—	254	444	144
A. soyogo	182	258	670	4	93	—	—	46	130	121	276

[a] Bt: *Bracon tamabae*; Me: *Mesopolobus* sp.; To: *Torymus* sp. [b] In: *Inostemma* sp.; Ga: *Gastrancistrus* sp. [c] Unknown parasitoids include both larvae and exuvium of parasitoids. [d] Unknown includes empty chambers from which midge and parasitoid adults have escaped.

DISCUSSION

Our analysis of the number of chambers in the gall and gall diameter indicated that rates of gall-size-dependent parasitism by ectoparasitoids are similar between the two midges. Simple comparisons of clutch size among species are problematic, because clutch size differences may be correlated with several ecological, morphological and life-history variables (Godfray *et al.*, 1991). In *A. sasakii* and *A. soyogo*, however, the life-history variables are quite similar. Moreover, the size of the galls on *I. crenata* and *I. integra* did not differ if the larval densities in the galls were the same (Fig. 1). Therefore, our methods have a logical basis in the detection of differences in the behavioural responses of gall-forming insects to attack by their natural enemies.

In both midge species, the rate of attack by parasitoids was density dependent (i.e. dependent on the number of larvae present), and this effect changed throughout gall formation: before the growth phase of the gall, the rate of endoparasitoid attack increased with increasing larval density; after gall maturation, the rate of ectoparasitoid attack decreased with increasing larval density (see below).

Endoparasitoids specific to each midge showed a density-dependent parasitism (Fig. 3B). The two endoparasitoids, *Inostemma* sp. for *A. sasakii* and *Gastrancistrus* sp. for *A. soyogo*, attack midge eggs before the growth phase of the gall (Tabuchi and Amano, 2003a). This suggests that the rate of parasitism by endoparasitoids is affected by the density of midge eggs in the axillary buds, not by the size of the gall, because the gall grows long after the attack by parasitoids. Some of the endoparasitoid larvae suffered death by hyperparasitism by ectoparasitoids, so we did not clarify the net rate of parasitism by endoparasitoids. However, it is unlikely that ectoparasitoids attacked large numbers of midge larvae containing larval endoparasitoids, so our observations of the rates of attack by both endoparasitoids may be reliable.

For both midge species, ectoparasitoid attack caused an inverse density-dependent mortality in the larvae inhabiting the gall (Fig. 3A). The attacking behaviour of ectoparasitoids may be influenced mainly by the size of available galls: it is impossible for the ectoparasitoids to attack and oviposit on host larvae in the centres of large galls. For other gall-making insects in multilocular galls, it is known that a large gall containing many larvae provides a structural refuge against parasitoids that attack after gall maturation (Jones, 1983; Weis *et al.*, 1983; Zwölfer and Arnold-Rinehart, 1994; Freese and Zwölfer, 1996). A similar result has also been reported in both galling and non-galling phytophagous species: large unilocular galls and plant parts such as bark, flower heads, stems and trunks provide physical refuge for endophytic insect herbivores (Ball and Dahlsten, 1973; Gargiullo and Berisford, 1981; Price and Clancy, 1986; Romstöck-Völkl, 1990; Freese, 1995).

Rates of attack by ectoparasitoids differed significantly between the two midges. In larger galls, *A. sasakii* was more frequently attacked by ectoparasitoids than *A. soyogo* (Fig. 3A). This result may have been caused by differences in the ectoparasitoid fauna of the two midge species. Two of the three ectoparasitoids, *Bracon tamabae* and *Mesopolobus* sp., assault both midges, and the third species, *Torymus* sp., attacks only the larvae of *A. sasakii* (Tabuchi and Amano, 2003a). *Bracon tamabae* has a relatively long ovipositor (~1.44–2.88 mm: see Maetô, 1991), which makes it possible to attack larvae in large galls. *Torymus* sp. also has a long ovipositor (~5 mm: K. Tabuchi, personal observation). In contrast, *Mesopolobus* sp. has a short ovipositor (K. Tabuchi, personal observation). In light of these differences in ovipositor length, the gall size range suitable for attack by *B. tamabae* and *Torymus* sp. may be wider than that for *Mesopolobus* sp. In addition, *Torymus* sp. attacks only *A. sasakii*, and its attacking performance might therefore play an important role in the observed heterogeneity of parasitism (Fig. 3A). The differences in parasitoid fauna (Table 4) also suggest that the major ectoparasitoid affecting the larval survival of *A. soyogo* is *Mesopolobus* sp., whereas in *A. sasakii* both *Mesopolobus* sp. and *Torymus* sp. play important roles.

Parasitism produced bell-shaped curves relating survival rates to numbers of larvae in each gall (Fig. 4). The number of chambers per gall with maximum larval survival was determined by the balance of a series of parasitisms in both midges, and especially by the antagonistic forces of endo- and ectoparasitoids. Survivorship peaks of both midges varied in each season with the density and diversity of parasitoid fauna (Table 3, Figs. 3 and 4). The optimum number of larvae (and therefore chambers) in the galls of *A. sasakii* was higher than in *A. soyogo* (Fig. 4). The mean number of chambers in each gall of *A. sasakii* was also higher than in *A. soyogo* (Fig. 2), and we suggest that *A. sasakii* adult females deposited more eggs at each oviposition site than did *A. soyogo*. Therefore, natural enemies have exerted selection pressures of different intensities on the two species and have introduced biological and evolutionary differences in the number of chambers (and, presumably, clutch size). Indeed, Tabuchi and Amano (2003b) showed that *A. sasakii* adult females had more eggs in their ovaries (169.7 ± 11.4) than did *A. soyogo* (136.4 ± 8.0) ($P < 0.05$ by *t*-test), suggesting a higher oviposition potential in *A. sasakii*.

In both midges, the mean number of chambers per gall was smaller than the optimum number of chambers required for maximal survival (Figs. 2 and 4). One explanation for this discrepancy may be that some larvae died before chamber formation, owing to failure in hatching and other reasons. This sort of discrepancy has been reported in another gall-fly, *Urophora cardui*, on *Cirsium arvense* (Freese and Zwölfer, 1996). An alternative explanation for the discrepancy may be the constraint of time: the adult females of *A. sasakii* and *A. soyogo* in our study could not deposit their full capacities of eggs in large clutches as a result of their short lifespans. In general, cecidomyiid adults live only one or a few days (Barnes, 1956; Redfern, 1975; Sunose, 1983; Miller and Borden, 1984; Yukawa, 2000). Tabuchi and Amano (2003b) noted that the lifespan of captive, non-breeding adults of *A. sasakii* and *A. soyogo* is 4 or 5 days in the laboratory and that their longevity in the field might be shorter. Considering that one of the major mortality factors for the adult midge is predation (Weis *et al.*, 1983; Yukawa, 1983), the long time taken for females to oviposit large clutch sizes may not be an adaptive trait. We observed hunting spiders capturing midge adults of both species that perched on the leaves of the host plant. This may be another factor selecting for smaller clutch size in the two midges.

In summary, our results indicate that the numbers of chambers in the galls of *A. sasakii* and *A. soyogo* were significantly different: the galls of *A. sasakii* usually contained more larvae than those of *A. soyogo* (Fig. 2). This difference is correlated with the heterogeneity of attack by natural enemies and parasitoids (Tabuchi and Amano, 2003a). Therefore, we conclude that in these two closely related gall midges, different pressures by parasitoid attack leads to different directional selection of the numbers of chambers in each gall and therefore different oviposition strategies of females.

ACKNOWLEDGEMENTS

We thank Dr K. Ozaki (Hokkaido Research Center of Forestry and Forest Products Research Institute) for his critical reading of an early draft of the manuscript.

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