

Assessment of hybrid vigor between flightless lines to restore survival and reproductive characteristics in the ladybird beetle *Harmonia axyridis*

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Abstract The effectiveness of hybrid vigor, which can counteract deleterious effects of inbreeding in flightless *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), was investigated. First, we performed the reciprocal cross between two isofemale lines and compared survival and reproductive characteristics between isofemale and hybrid lines under laboratory conditions. The survival of one of the hybrid lines was significantly higher than that of the two isofemale lines. Early fecundity tended to be higher in the two hybrid lines than in the two isofemale lines. Second, we compared the effectiveness of control of *Aphis gossypii* Glover (Homoptera: Aphididae) between isofemale and hybrid lines by release experiments in greenhouses. The number of *A. gossypii* was suppressed in treatments in which two hybrid lines were released compared with those in which two isofemale lines were released. These results suggest that hybrid vigor is effective as a method for assuring the quality of flightless *H. axyridis*.

Keywords Biological control · *Harmonia axyridis* · Flightless · Inbreeding depression · Reciprocal cross · Quality control

Introduction

Inbreeding depression accumulates over generations, and does so more rapidly in smaller populations (Miyatake 1998, 2011; Frankham et al. 2002). Characteristics most closely related to reproductive fitness show greater inbreeding depression than those that are peripherally related to fitness (Mousseau and Roff 1987; Falconer and Mackay 1996; Lynch and Walsh 1998). The effects of inbreeding have been described as potential deterrents to genetic improvement projects of biological control agents (Hoy 1985). Ladybirds have been widely recognized as biological control agents for aphids (Dixon 2000; Obrycki et al. 2009). However, inbreeding has been reported to cause a severe loss of fitness in some species (Kidd 1993; Majerus 1994; Hurst et al. 1996; Morjan et al. 1999; Benoît et al. 2011).

The use of ladybirds in augmentative biological control has been hampered by the tendency of adults to disperse (Hagen 1962; Marples et al. 1993; Dreistadt and Flint 1996; Dixon 2000). Flightless lines have been established for *Harmonia axyridis* (Pallas) (Ferran et al. 1998; Tourniaire et al. 2000; Seko et al. 2008) and *Adalia bipunctata* L. (Marples et al. 1993; Ueno et al. 2004; Lommen et al. 2008). These

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lines have been demonstrated to be effective for augmentative biological control of aphids (Weissenberger et al. 1999; Seko et al. 2008; Lommen et al. 2008; Adachi-Hagimori et al. 2011).

Artificial selection tends to erode genetic variation (Falconer and Mackay 1996; Roff 1997), and the loss of genetic diversity is directly related to the level of inbreeding (Frankham et al. 2002). In some species of ladybirds, flightlessness can be controlled by homozygosity for the recessive allele at one major locus (Marples et al. 1993; Ferran et al. 1998; Tourniaire et al. 2000; Ueno et al. 2004). Inbreeding increases the frequency of homozygotes, and may expose deleterious recessive alleles (Hoy 1985; Hopper et al. 1993; Falconer and Mackay 1996; Roff 1997; Lynch and Walsh 1998). These factors can lead to reduced fitness in terms of development and reproduction of flightless ladybird beetles. Seko and Miura (2009) found that artificial selection for reduced flight ability can induce inbreeding depression on developmental and reproductive trait of *H. axyridis*. To be successful in the mass rearing and biological control of aphid populations, the genetics of the flightless *H. axyridis* should be managed for quality assurance.

One way to ensure that alleles will not be lost to selection might be to make them completely homozygous (fixed) in small colonies, preferably in isofemale lines (Roush 1990; Roush and Hopper 1995; Falconer and Mackay 1996). Although this would induce deleterious effects of inbreeding for each isofemale line, hybrid vigor between these lines should restore genetic variation (Hopper et al. 1993). In this study, we investigated the effectiveness of hybrid vigor, to counteract deleterious effects of inbreeding in flightless lines of *H. axyridis*. First, we performed the reciprocal cross between two isofemale lines and compared fitness parameters between isofemale lines and hybrid ones under laboratory conditions. Second, we compared the effectiveness for control of the cotton aphid *Aphis gossypii* Glover between isofemale and hybrid lines by carrying out release experiments in greenhouses.

Materials and methods

Establishment of flightless lines

Figure 1 provides an overview of the experimental work in this study. A flightless line of *H. axyridis* was

established by artificial selection for reduced flight ability on a laboratory population from 80 adults of *H. axyridis* that were collected in Fukuyama (Western Japan, Lat. 34°28'N, Long. 133°23'E) during late April 2003 at NARO Western Region Agricultural Research Center, Japan (see Seko et al. 2008). Thirty egg clusters of *H. axyridis* were collected, and 130 hatchlings were randomly selected from these egg clusters (the first generation). After emergence, 48 adults of each sex were measured for flight distance using the flight mill system (Seko et al. 2008). Adults that recorded shorter flight distances (bottom 30%) were selected and mated in plastic square containers. Egg clusters were collected from selected adults. The same procedure as described above was repeated in the next generation. An isofemale line was established from selected adults from the seventh generation to promote homozygosity. Four adults that recorded shorter flight distances (bottom 25% in selected adults) from each sex were selected, and four mating pairs were established. Only one of the four mating pairs succeeded to produce offspring. Though flight distance was not measured because the population size was less than ten individuals during the eighth and ninth generations, the selection was resumed from the tenth generation onward. The procedure and population size per generation were the same as those before the seventh generation. This line is referred to as the isofemale line A. Because of a lack of food in the laboratory, the strain was maintained with a population size of 24 males and 39 females in the fourth generation, and 25 males and 20 females in the thirteenth generation with no artificial selection.

Another isofemale line B was established by the following procedure. Eighty adult *H. axyridis* were collected in Fukuyama during mid-April 2004 and were allowed to mate and lay eggs on tissue paper at a density of 40 individuals per square plastic container (16 × 22 × 8 cm). Thirty egg clusters were collected and from these, 130 hatchlings were randomly selected. Following emergence, the artificial selection was also performed like for the isofemale line A.

Because of a lack of food in the laboratory, line B was maintained with a population size of 30 males and 30 females for the 13th generation and 24 males and 24 females for the 19th generation. Isofemale lines were established from selected adults from the 27th generation to promote homozygosity.

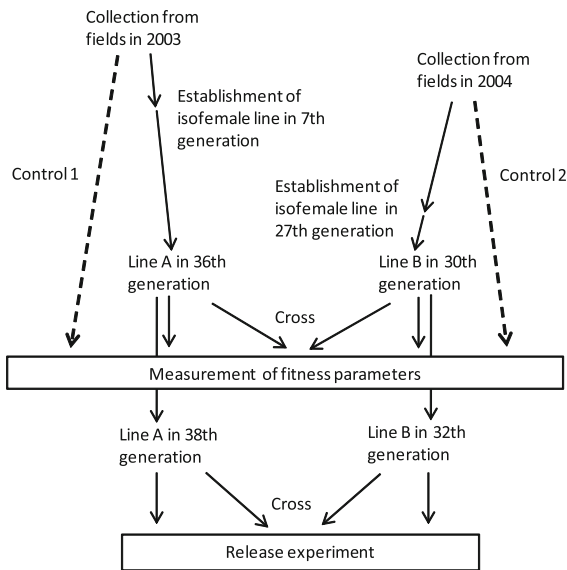


Fig. 1 Flow chart of the establishment of isofemale and hybrid lines used in the present study. Solid and dashed arrows show that artificial selection and no artificial selection were conducted on flight ability of *H. axyridis*, respectively

Flight distances were measured using flight mills. Twelve flight mills were run simultaneously during the experiment. A piece of balsawood ($0.5 \times 0.1 \times 10$ cm, 0.23 g) was used as a flight mill rotor. A small amount of bond (Zero time wide, Loctite, Ltd., Tokyo, Japan) was applied to the left elytra of 48 adults of each sex. Each adult was attached to the tip of the flight mill rotor. After that, all adults started to flap their wings immediately. They were allowed to fly for 1 h under light. When a light beam that was aimed vertically through each rotor was interrupted by the revolution of flight mill rotor, the time of occultation was automatically logged in the computer unit (KV-700, Keyence, Ltd., Osaka, Japan). From the time of every occultation on each channel, the total number of revolutions and the duration of each revolution were calculated.

Seven adults that showed shorter flight distances (the lower 25% of selected adults) from each sex were selected and seven mating pairs were established. In one of them, the flight distance in 1 h was nearly 0 m from the 28th generation onward. The selection on flight ability of *H. axyridis* for one of the seven families was resumed from the 28th generation onward. The population size per generation was 36 males and 36 females. Non-flying adults of the

isofemale line B had normal elytra and wings, but the rotor of the flight mill system did not spin because the ladybirds could not flap their wings.

Control 1 (see Seko et al. 2008) and control 2 from the same adults that were used to establish the isofemale line A and B, respectively, were maintained with randomly selected offspring, thereby imposing no artificial selection on flight ability. Twenty egg clusters of *H. axyridis* were collected, and 80 hatchlings were randomly selected from these egg clusters. After emergence, 40 adults (20 males and 20 females) were selected randomly and mated in plastic square containers. The same procedure as described above was repeated in the next generation.

Two types of hybrid lines were established by performing reciprocal crosses. Twenty-five males and 25 females from the isofemale lines A (the 36th generation) and B (the 30th generation), respectively, were allowed to mate and lay eggs to propagate the hybrid line A(m)B(f). Similarly, 25 males and 25 females from the isofemale lines B and A, respectively, were allowed to mate and lay eggs to propagate the hybrid line A(f)B(m). These lines were established under LD 16:8 at 25°C and 40–60% RH. In the laboratory, larvae and adults of *H. axyridis* were reared at a density of 20 individuals per circular plastic container (diameter 10 cm \times height 5.5 cm). They were fed daily on pea aphids *Acyrtosiphon pisum* Harris, which were raised on broad bean seedlings in plastic cages (26 \times 18 \times 13 cm).

Measurement of life-history traits and flight performance of each line

Several parameters of development and reproduction were measured for the two isofemale lines and the two hybrid. Egg clusters of the isofemale line B, control 2, and the two hybrid lines were placed on a piece of wet filter paper in a Petri dish (diameter 9 cm) until they hatched. The number of eggs in an egg cluster was counted prior to hatching. The number of hatchlings was counted per 12 h for two days after the first hatchling was confirmed. Hatchlings were removed immediately from the Petri dishes to avoid egg cannibalism given a lack of synchrony in hatching (especially in the isofemale lines). A random sample of 110–120 hatchlings in each line was individually introduced into a plastic cup (diameter 4.5 cm, height 3.5 cm) and fed cucumber leaves with *A. gossypii*

(50–70 first to third instars and 100–150 fourth instars and adults, respectively). A piece of wet filter paper was placed at the bottom of the cup. Food was replenished daily.

Pupae were weighed with a Sartorius 4503MP6E scale with an accuracy of 0.001 mg on the day following pupation. Developmental period (from hatch to adult emergence) was monitored on a daily basis and the proportion emergence was noted. Pairs of one male and one female were randomly established on the day following adult emergence. The preoviposition period of the females was monitored on a daily basis. Early fecundity was measured daily over the first ten days after the onset of oviposition.

Hybridization may affect not only development and reproduction, but also the flight ability of *H. axyridis*. Therefore, the flight distance of each line over a period of 1 h was measured. Rearing and measurement of flight ability was done as for the isofemale lines A and B. The flight distances of 30–32 adults of each sex were measured. The flight distance of isofemale line A was measured in the 36th generation. That of isofemale line B and control 2 was measured in the 30th generation. These experiments were conducted under LD 16:8 at 25°C and 40–60% RH. The data of the isofemale line A (except the flight distance) and control 1 were taken from Seko et al. (2008) and Seko and Miura (2009).

Release experiment

Five plots, i.e., for the two isofemale lines A and B, the hybrid lines A'(f)B'(m) and A'(m)B'(f), and no treatment, were used to evaluate the effects of hybrid vigor on the control potential of *H. axyridis* against *A. gossypii*. The plots were set up in five small-scale greenhouses with cultivated eggplants, *Solanum melongena* (L.). Hybrid lines A'(f)B'(m) and A'(m)B'(f) were established by performing reciprocal crosses between 25 males and 25 females from isofemale lines A (38th generation) and B (32th generation). The release experiment was carried out at NARO Western Region Agricultural Research Center, Japan from 3 May to 8 July 2008. Eggplant seedlings (cv. 'Chikuyô') with five leaves were transplanted on 23 April 2008. Four plots consisted of two rows of plants with 100 cm spacing between rows and 60 cm between plants so that each plot contained 20 plants. In one plot, on which the hybrid

line A'(f)B'(m) was released, 30 plants were transplanted because the area of this greenhouse was larger (5.5 × 20.2 m) than the other ones (5.3 × 13.0 m). The incidence of *A. gossypii* was observed in all treatments within one week after the transplantation. Seven-day-old adult ladybirds of each line were released on eggplant stocks with *A. gossypii* on May 13, 20, and 27, 2008. The release density was one adult per eggplant, i.e., for the line A'(f)B'(m): 15 males and 15 females, and for the other lines A, B and A'(m)B'(f): ten males and ten females. Numbers of *A. gossypii* and *H. axyridis* on eggplants were counted every three days. Six leaves were randomly selected per eggplant to count aphid numbers.

Statistical analyses

Statistical analyses were performed using JMP, version 9. Normally distributed variables for hatching rate (after arcsine transformation), pupal weight, and early fecundity were compared among lines using the Tukey–Kramer test. The normality of the data was assessed using the Shapiro–Wilk test. Non-normally distributed data for the developmental period, preoviposition period, and flight distance was compared using the Steel–Dwass test (Steel 1960). For the proportion emergence, after the χ^2 test was performed, the significance levels were subsequently adjusted according to the sequential Bonferroni correction method.

Analysis by two-way ANOVA was used to compare the incidence of *A. gossypii* among greenhouses with dates as replicates, treatment as a fixed effect, stock and leaf nested in treatment as a random effect. Stock is the primary sampling unit and leaf is the secondary sampling unit. Numbers of *H. axyridis* adults and fourth instar larvae per stock were also compared by the same procedure with dates as replicates, treatment as a fixed effect, and stock nested in treatment as a random effect.

Results

Measurement of performance in hybrid lines

Table 1 shows the differences in viability among the studied lines of *H. axyridis*. The hatching rate of the hybrid line A(m)B(f) was significantly higher than that

of the two isofemale lines. The proportion emergence was also higher in the hybrid line A(m)B(f) than in the two isofemale lines. Hatching rate and proportion emergence of the other hybrid line A(f)B(m) did not differ from that of the two isofemale lines. Further, pupal weight did not differ between the isofemale and hybrid lines in both sexes and early fecundity of the hybrid line A(m)B(f) was significantly higher than in the two isofemale lines (Table 1). The developmental period of the control 1 and the hybrid line A(m)B(f) was shorter than that of the isofemale line A in both sexes (Table 2). The preoviposition period did not differ between the isofemale and hybrid lines (Table 2). The flight distance of isofemale and hybrid lines did not differ in both sexes, but was lower than that in the controls 1 and 2 (Table 3).

Release experiment

Figure 2 shows the incidence of (a) *A. gossypii*, (b) adults of *H. axyridis*, and (c) fourth instar larvae of *H. axyridis*. For the number of *A. gossypii*, two-way ANOVA was significant in all terms (treatment: $F_{4,95} = 57.0$, $P < 0.0001$; stock: $F_{95,500} = 9.2$, $P < 0.0001$; leaf: $F_{500,10115} = 2.0$, $P < 0.0001$; dates: $F_{17,10115} = 681.7$, $P < 0.0001$; dates \times treatment: $F_{68,10115} = 47.3$, $P < 0.0001$). The number of *A. gossypii* reached about 400 individuals per leaf in early June in the no treatment greenhouse. Thereafter, the number of *A. gossypii* in the no treatment greenhouse decreased because many leaves of egg-plant stocks suffered heavy damage, caused by the outbreak of *A. gossypii*. The number of *A. gossypii* was

lower in treatments in which adults of the hybrid lines were released than in those in which adults of the isofemale lines were released. In the plots of two hybrid lines, the incidence of fourth instar larvae, i.e., the offspring of released adults, was greater than that in the plots with the isofemale lines. For the numbers of adult *H. axyridis*, two-way ANOVA was significant in all terms (treatment: $F_{3,86} = 3.3$, $P < 0.05$; stock: $F_{86,1204} = 2.9$, $P < 0.0001$; dates: $F_{14,1204} = 14.1$, $P < 0.0001$; dates \times treatment: $F_{42,1204} = 1.9$, $P < 0.001$). For the numbers of fourth instar larvae of *H. axyridis*, it was significant in terms of treatment ($F_{3,86} = 9.2$, $P < 0.0001$), dates ($F_{14,1204} = 19.7$, $P < 0.0001$) and date \times treatment ($F_{42,1204} = 5.3$, $P < 0.0001$), but not in terms of stock ($F_{86,1204} = 1.2$, $P > 0.05$).

Discussion

Commercial populations can lose genetic diversity through genetic drift (founder effects, bottleneck effects) and unintentional selection during mass production (Hopper et al. 1993; Fernández and Nentwig 1997; Honda et al. 2004; Hinomoto et al. 2006). Since the traits related to commercial production generally show strong inbreeding depression (Falconer and Mackay 1996), the suppression of increased inbreeding in the commercial product will be an important issue. Hybridization programs for avoiding the loss of alleles have been discussed (Hoy 1976; Beckendorf and Hoy 1985; Hoy 1985; Roush and Hopper 1995; De Clercq et al. 1998). Ueno et al.

Table 1 Hatching rate, proportion emergence, pupal weight and fecundity of different lines of *H. axyridis*

Line	n^a	Hatching rate (median (min.–max.))	n	Proportion emergence	Pupal weight (mean \pm SD) (mg)				n	Early fecundity (mean \pm SD) ^b
					n	Male	n	Female		
A	46	0.40 (0–1) a	110	0.51 a	35	32.4 \pm 4.0 a	21	39.4 \pm 4.7 a	16	103.3 \pm 53.1 a
B	46	0.50 (0–1) a	120	0.48 a	30	33.4 \pm 3.8 a	27	39.7 \pm 4.0 a	15	133.6 \pm 73.4 ab
A(f) B(m)	37	0.54 (0–1) ab	118	0.43 a	26	33.4 \pm 4.4 a	25	37.3 \pm 4.4 a	14	192.6 \pm 90.2 bc
A(m) B(f)	45	0.80 (0–1) c	110	0.70 b	32	32.9 \pm 4.3 a	45	38.1 \pm 4.9 a	23	216.6 \pm 103.5 c
Control 1	46	0.93 (0–1) c	99	0.72 b	45	36.4 \pm 3.0 b	26	40.7 \pm 3.7 a	24	193.0 \pm 90.3 bc
Control 2	47	0.74 (0–1) bc	60	0.72 b	25	34.3 \pm 4.7 ab	22	39.4 \pm 3.5 a	19	211.2 \pm 66.0 bc

^a Number of egg clusters

^b Early fecundity (expressed as no. eggs per female) was measured over the first ten days after the onset of oviposition

Data of line A and control 1 were from a previously published study (Seko and Miura 2009)

Values within a column followed by different letters are significantly different ($P < 0.05$)

Table 2 Developmental and preoviposition period (in days) of different lines of *H. axyridis*

Line	Developmental period (median (min.–max.))				<i>n</i>	Preoviposition period (median (min.–max.))
	<i>n</i>	Male	<i>n</i>	Female		
A	35	16.0 (11.0–19.0) a	21	15.0 (14.0–20.0) a	16	10.0 (7.0–20.0) a
B	30	15.0 (13.0–18.0) ab	27	15.0 (14.0–18.0) ab	15	8.0 (6.0–16.0) ab
A(f) B(m)	26	15.0 (14.0–18.0) ab	25	15.0 (13.0–18.0) ab	14	9.0 (6.0–19.0) ab
A(m) B(f)	32	15.0 (14.0–18.0) b	45	14.5 (13.0–16.0) b	23	9.0 (6.0–18.0) ab
Control 1	45	15.0 (13.0–19.0) b	26	14.5 (13.0–16.0) b	24	7.5 (4.0–17.0) b
Control 2	25	15.0 (14.0–16.0) ab	22	14.5 (13.0–17.0) b	19	10.0 (4.0–17.0) a

Medians within a column followed by different letters are significantly different ($P < 0.05$, Steel–Dwass test). Data of line A and control 1 were from a previously published study (Seko and Miura 2009)

Table 3 Flight distance for 1 h (median (min.–max.), in m) of males and females from different lines of *H. axyridis*

Line	<i>n</i>	Male	<i>n</i>	Female
A	32	0 (0–33.3) a	32	0 (0–15.7) a
B	30	0 (0–1.4) a	30	0 (0–12.6) a
A(m) B(f)	30	0 (0–3.6) a	30	0 (0–0.5) a
A(f) B(m)	30	0 (0–1050.8) a	30	0 (0–363.5) a
Control 1	30	54.1 (0–2137.9) b	30	173.5 (3.6–1868.5) b
Control 2	30	192.9 (0–2187.9) b	30	518.3 (0.3–1651.6) b

Medians within a column followed by different letters are significantly different ($P < 0.05$, Steel–Dwass test). The flight distance of the lines A and B was measured in the 36th and 30th generations, respectively. Data of control 1 were from a previously published study (Seko et al. 2008)

(2004) suggested that the wingless gene has strong negative pleiotropic effects on certain fitness components in *A. bipunctata*. Hybridization should be effective to reduce the loss of fitness by inbreeding depression, but not be effective to reduce fitness loss due to pleiotropic effects. In our study, viability of a hybrid line was significantly greater than that of two isofemale lines in flightless *H. axyridis*. Early fecundity tended to be higher in two hybrid lines than in two isofemale lines. The number of *A. gossypii* was suppressed in greenhouses in which two hybrid lines were released compared with those in which two isofemale lines were released. These results suggest that hybrid vigor between isofemale lines is effective as a method for assuring the quality of flightless *H. axyridis*.

Progeny of hybrid A(m)B(f) showed a higher hatching rate and proportion emergence than the alternative reciprocal cross. In the release experiment, the incidence of fourth instar larvae of the hybrid line A'(f)B'(m) was lower than that of A'(m)B'(f). These findings might indicate a lower viability of the hybrid

lines where females of isofemale line A were crossed with males of isofemale line B. The differential viability might be related to sex-linked inheritance (Kotze and Sales 2001; Tapia-Perez et al. 2003) or extra-nuclear components such as maternal effects (Geden et al. 1992; Roff and Sokolovska 2004). The influence of maternal effects on the growth and survival of progeny has been discussed (Mousseau and Dingle 1991; Roff 1992; Mousseau and Fox 1998). Differences in fecundity between reciprocal crosses have also been reported in parasitoids (Geden et al. 1992; Baya et al. 2007). Fecundity of F₁ hybrids resembled their mothers more than each others, indicating that it was partially under nonchromosomal maternal control (Geden et al. 1992). The amount and quality of resources allocated to propagules by females might differ between the isofemale lines A and B. Early fecundity of A(f)B(m) did differ from that of the isofemale line A, but not from that of the isofemale line B. Inbreeding depression may not have affected isofemale line B in the same way it affected isofemale line A, given that isofemale line A was

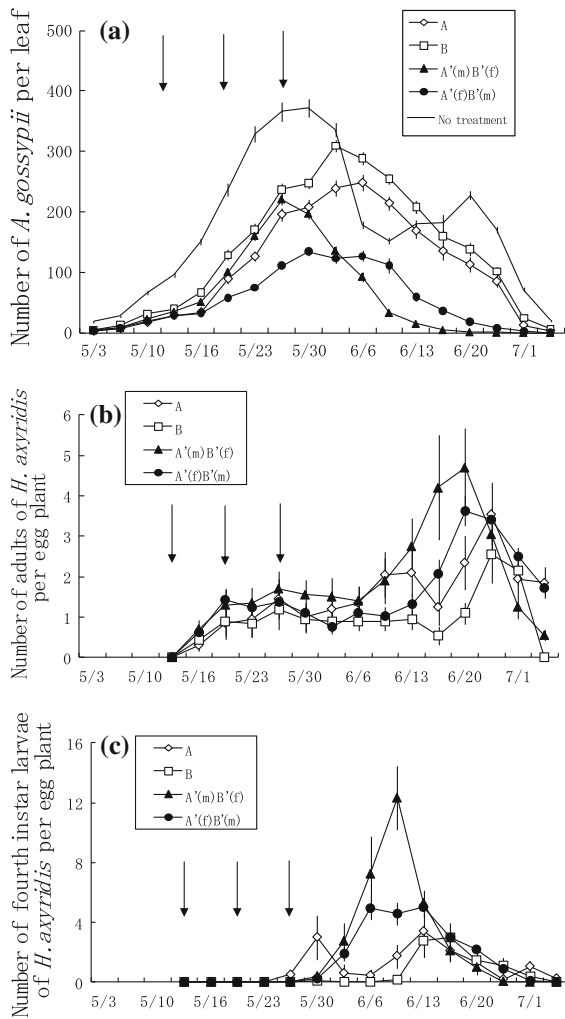


Fig. 2 The incidence of (a) *A. gossypii*, (b) adults of *H. axyridis*, and (c) fourth instar larvae of *H. axyridis* in plots in which eggplants were cultivated in small-scale greenhouses. All values are expressed as means \pm SE. Arrows show the day on which *H. axyridis* adults were released in each plot

established earlier than isofemale line B. Although fecundity of isofemale line B did not statistically differ from that of control 1 and 2, it might possibly decrease in future generations by progressing inbreeding.

Our results support the hypothesis that the restoration of fitness parameters such as viability and fecundity in flightless *H. axyridis* can reflect the effectiveness for control of aphids. Hybrid vigor could restore not only viability and fecundity but also voracity and/or searching behavior. Walking activity

levels during the daytime in both sexes of flightless strains of *H. axyridis* tended to be lower than those of the control, but the reduction in walking activity was not restored by hybrid vigor (Nakayama et al. 2010). Latter authors proposed that this reduction might not be due to inbreeding depression but to a genetic correlation between flight ability and walking activity. Numbers of prey consumed did not differ between flightless and control strains at almost all developmental stages (Nakayama et al. unpublished). Higher numbers of fourth instar larvae of *H. axyridis* were observed in the greenhouses with either hybrid line than in those with the isofemale lines, suggesting that number of the offspring of released adults was the main factor controlling *A. gossypii*.

Roush and Hopper (1995) recommend the following mixed strategy for the genetic management of arthropods, especially for biological control agents: (1) use a few large colonies to avoid drift and inbreeding, maintained under conditions (such as variable environments) that might reduce inadvertent selection, and (2) use 25–50 independent isofemale lines to avoid loss of alleles. On the other hand, the management of many isofemale lines is likely to be difficult in terms of the cost and labor associated with mass rearing. Another possible method for restoring genetic variability in a laboratory line is to outcross wild populations to laboratory ones (Whitten and Foster 1975; Hoy 1976; Roush and Hopper 1995). This could be cost effective because the management of many isofemale lines would be unnecessary. Although in *H. axyridis* a regain of flight ability as well as the improvement of survival and reproductive characteristics can be expected, we should be able to partially separate flightless individuals after the F₂ generation. Further information on the manner of inheritance of flight ability is required to determine the effectiveness of outcrossing wild populations of *H. axyridis* to flightless laboratory ones.

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