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Author(s)	Akimoto, Shin-ichi; Murakami, Taiga
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title: Condition-dependent sex allocation by clones of a galling aphid

authors: Shin-ichi Akimoto · Taiga Murakami

Systematic Entomology, Department of Ecology and Systematics, Graduate School of
Agriculture, Hokkaido University, Sapporo, 060-8589, Japan

corresponding author: S. Akimoto (e-mail)

Systematic Entomology, Department of Ecology and Systematics, Graduate School of
Agriculture, Hokkaido University, Sapporo, 060-8589, Japan

e-mail: akimoto@res.agr.hokudai.ac.jp

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Abstract Local mate competition (LMC) has been postulated to be the primary factor of female-biased sex allocation. In animals such as aphids that exhibit seasonal alternations of clonal and sexual reproduction, there is a high possibility of intra-clonal mating and LMC. This possibility is more plausible for more fecund clones, but outbreeding is predicted for less fecund clones. We hypothesize that clones that are more fecund will gain higher fitness returns by reducing investment in males because of more intense LMC among clonal males. We tested this hypothesis by elucidating the clonal sex allocation patterns of the galling aphid *Kaltenbachiella japonica*, in which inbreeding and LMC appear to be common. Winged mothers that emerge from a gall, belonging to the same clone, produced males and sexual females asexually on a branch, without dispersing to other trees. The heavier the gall, the more winged mothers were produced from the gall. Individual mothers produced a constant number of males and a variable number of females. The clonal sex allocation to males was 39.8%, on average, and decreased with increasing gall weight. This result showed that clones that were more fecund exhibited more female-biased sex allocation and thus supported our hypothesis. Furthermore, our results corroborated Stubblefield and Seger's hypothesis for sex allocation in patch structure rather than Yamaguchi's constant male hypothesis. We conclude that *K. japonica* clones are able to adjust their sex allocation patterns adaptively depending on the quality of resources in the galls.

Keywords Inbreeding · *Kaltenbachiella japonica* · Local mate competition · Local resource competition · Parthenogenesis · Sex ratio

Introduction

The population sex ratio is affected by several population traits (Wrensch and Ebbert 1993; Hardy 2002; West 2009). Local mate competition (LMC) (Hamilton 1967) and local resource competition (LRC) (Clark 1978) are the main population mechanisms that lead to deviation from the sex ratio of 1:1, at which an evolutionary equilibrium is maintained in randomly mating populations (Fisher 1930); as the population sex ratio approaches 1:1, maternal fitness returns from producing sons is balanced with the fitness returns from producing daughters. However, if sons compete for mates, female-biased sex allocation could evolve in populations because mothers gain greater reproductive returns by economizing investment in their sons (Hamilton 1967). By contrast, where daughters compete with each other for resources, male-biased sex allocation could evolve (Clark 1978). These competitive situations among siblings are partly attributable to the structure of the population. When a breeding population is composed of small patches in which foundress mothers reproduce, competition among sons or daughters is expected, depending on whether sons remain in the patch with daughters dispersing to new patches after copulation, or if daughters remain in the patch with sons dispersing to new patches (Clark 1978; Werren 1983; Bulmer 1986; Molbo and Parker 1996; Herre et al. 1997; Perrin and Mazalov 2000; Cockburn et al. 2002).

In addition to these factors, individual mothers may have the ability to control their sex allocation depending on the environmental conditions they confront. One condition is the reproductive status of the mother. Trivers and Willard (1973) predicted that mothers with good reproductive resources would invest more in the sex that derives higher reproductive success from a given level of investment. In most mammals, males vary more in reproductive success; thus, increased maternal investment in sons

possibly contributes positively to the reproductive success of sons. In this situation, mothers would gain higher fitness returns by increasing their investment in respective sons than in respective daughters. Thus, this hypothesis predicts that mothers with good reproductive resources should invest more in sons, whereas mothers with poor resources should invest more in daughters.

Almost all empirical and theoretical studies have focused on individual-level sex allocation and predicted the optimal allocation strategy for each foundress mother (as an exception, see McGovern 2002). However, for animals such as aphids that exhibit seasonal alternations of clonal and sexual reproduction, it is necessary to evaluate the sex allocation patterns of clones in a population because clones are the targets of natural selection. Regarding sex ratios in aphids, Moran (1993) pointed out that predictions of allocation theory apply to the ratio produced by the co-occurring members of a clone rather than the ratio produced by individual mothers.

Organisms that include a stage of clonal reproduction are characterized by the possibility of clonal self-fertilization unless they have a high ability to disperse (Knowlton and Jackson 1993; Eckert 2000). Successful clones producing more members prior to sexual reproduction would have a higher possibility of mating between members of the same clone, whereas clones producing fewer members would be more likely to outbreed because they are surrounded by members of other clones (Eckert 2000; Albert et al. 2008). In cases where intensive inbreeding is possible, there is ample evidence indicating that LMC among clonal or related males is likely to arise simultaneously (Alexander and Sherman 1977; Werren 1980; Hasegawa and Yamaguchi 1995; Abe et al. 2003; Schrempf et al. 2005). Thus, if clones are not well mixed during dispersal, it is predicted that clones that are more fecund could acquire higher inclusive fitness by reducing sex allocation to males to avoid LMC. This verbal model

substantially corresponds to Yamaguchi's model (1985) or Stubblefield and Seger's model (1990), although these models focus on individual mothers with different reproductive resources. Yamaguchi (1985) predicted that with increasing amounts of available resources, a mother should produce a constant number of sons and an increasing number of daughters under LMC (see Frank 1987 for detailed explanation). In contrast, Stubblefield and Seger (1990) expected an increasing number of sons as well as daughters in their model IIa. Furthermore, this model predicted that for any patch size, with increasing maternal investment, the absolute investment in sons increases more slowly than does the absolute investment in daughters (Stubblefield and Seger 1990, Figure 3). Yamaguchi's model assumes that when producing sexuals, foundress mothers have the complete knowledge about the level of their own reproductive resources, the number of foundresses in the patch, and the average level of reproductive resources of other foundresses (May and Seger 1985; Kindlmann and Dixon 1989; Stubblefield and Seger 1990). On the other hand, Stubblefield and Seger's "self-knowledge, random mating" model assumes a situation where foundress mothers know only their own reproductive resources, but do not know the number of foundresses in the patch or the average level of reproductive resources of other foundresses. Stubblefield and Seger's model, which seems to be based on a more realistic situation, has remained untested so far (Moore et al. 2005).

In the present study, we first aimed to test these hypotheses by using an aphid species with clonally reproducing generations before sexual reproduction. To evaluate clonal sex allocation, it is required to count the number of males and sexual females that are produced by every mother of the same clone. However, most previous studies on aphids have used host-alternating species as the material (Moran 1993; Foster 2002; Akimoto and Yamaguchi 2004); thus, it was difficult to estimate the sex allocation

patterns of the members of the whole clone because they dispersed to many plant individuals where they produced sexuals. On the other hand, in some aphid species, clonal members do not migrate to other host plants; they compete with other clones for reproductive success on the same host plant. In such species, selection will be exerted on the total number of sexuals (males and sexual females) produced by a single clone and the adjustment of the sex allocation. In this case, because clones send their sexuals to a single patch on the host individual, each clone, instead of individual mothers, could be regarded as a foundress in the patch. Therefore, by using sedentary aphid species, we can quantify clonal sex allocation patterns and test the two theories for sex allocation.

The present study focused on the eriosomatine, galling aphid *Kaltenbachiella japonica* (Matsumura, 1917) that exhibits a non host-alternating life cycle on the host plant, *Ulmus davidiana* var. *japonica* Nakai. This aphid is characterized by a short-cut life cycle with only 3 generations every year (Blackman and Eastop 1994). The first parthenogenetic generation induces closed galls, from which the second-generation winged females emerges to produce males and sexual females. A previous study has shown that winged females emerging from a gall migrated less than 1 m by walking to branches of the same host tree on which the gall was induced and rarely dispersed to other *U. davidiana* trees (Komatsu and Akimoto 1995). A feature resulting from this sedentary life cycle is a potentially high frequency of inbreeding and LMC. Approximately 100 clonal winged females from a single gall simultaneously produce males and sexual females parthenogenetically in a “patch” on large branches. Winged females of different clones join the patch if they emerge at the same time from the nearby galls. Thus, the mating system of a clone appears to be a mixture of self-mating and outbreeding. Furthermore, on each elm tree, the *K. japonica* population lasts for more than 10 years (S. Akimoto,

unpublished data). In some years, gall density radically decreases, but thereafter gall population propagates again probably through inbreeding. Thus, the breeding population is likely to be viscous. In fact, it is easy to obtain viable foundresses from enforced intracolonial self-mating (Komatsu and Akimoto 1995; S. Akimoto, unpublished data).

There are several advantages in using *K. japonica* as the material. First, it is possible to evaluate the sex allocation pattern of a whole clone precisely because each clone develops only within a single gall and produces sexuals. The sexuals develop fully within their winged mother and do not feed after they are born viviparously; thus, maternal sex allocation can be easily evaluated by counting the number of male and female embryos and by evaluating the costs incurred during their development (Akimoto et al. 2012). Second, it is possible to evaluate population sex allocation based on a large sample size by collecting many mature galls. Third, clonal sex allocation can be understood in connection with the resource level that a clone can use in the gall; we can measure gall mass as the index of the resource level in each gall. Fourth, it is possible to examine the proximate mechanism of sex allocation by observing embryos and ovarioles that each mother contains in her abdomen.

Therefore, the specific objectives of the present study were (1) to assess the sex allocation patterns at individual and clonal levels in *Kaltenbachiella japonica* in connection with resource levels in galls, (2) to test Yamaguchi's (1985) and Stubblefield and Seger's (1990) hypotheses based on the data of the clone-level sex allocation, and (3) to evaluate the proximate mechanism affecting the production of male and female embryos by each mother. On the basis of these data, we attempted to elucidate the sex allocation strategy by each aphid clone under changing resource conditions.

Materials and methods

Life cycle

K. japonica is an eriosomatine species that induces closed galls on the leaves of *Ulmus davidiana* var. *japonica* (Akimoto 1985). Aphid species of Eriosomatinae are typically characterized by a host-alternating life cycle, being associated with 2 kinds of host plant: the primary host plant on which galls are induced in spring and sexual reproduction occurs in autumn, and the secondary host plant on which aphids repeat parthenogenetic reproduction from early summer to autumn. However, *K. japonica* is not host alternating and is associated only with the Japanese elm *Ulmus davidiana* var. *japonica*, on which this species passes through 3 generations from spring to summer. First instars of the foundress generation hatch from overwintered eggs deposited on large branches in late April, and move to developing leaves, where they settle along the midrib to induce galls. Because first instars have a low ability to move, galling sites concentrate on leaves near the oviposition sites on main branches (Aoyama et al. 2012). As a result, more than 1 gall is frequently induced on 1 leaf. If 2 or more foundresses start gall induction in close vicinity along the same midrib, developing galls are fused, forming a “communal” gall where 2 or more foundresses coexist. *K. japonica* galls are completely closed with a hollow inside, in which foundresses parthenogenetically produce approximately 100 larvae. Except for communal galls, each gall represents a pure clone. The second generation develops into winged females that appear outside of the cracked galls from mid- to late-July and move to the main branches by walking. Winged females usually do not disperse by flight, although they can glide using their wings (Komatsu and Akimoto 1995). A number of winged females that appear from 1

or more galls gather on the rough surface of main branches and simultaneously deposit the third generation of males and sexual females. Males and sexual females mature sexually in a few days without feeding, and each sexual female deposits only a single egg in a crevice of the main branch after copulation. The eggs remain on the branches from early August until they hatch the next spring.

To avoid confusion about generations, the first generation is hereafter called foundresses, the second generation is called winged mothers, and the third sexual generation is called males and females (or male and female embryos in winged mothers) (Fig. 1).

Gall collection and counting of embryos

Galls were collected from one lower branch, about 15 cm in diameter at the base, of an elm tree on the campus of Hokkaido University on July 15, 2010. This branch was selected because a high gall density was observed on it and because mating and oviposition occurred on a lower branch. When the galls were collected, they remained unopened. Galled leaves were collected haphazardly from the branch, and the positions of the galled leaves on a shoot were not recorded. Collected galls were maintained for 4 days at room temperature to advance larval development, and then preserved in a container of 80% ethanol until they were analyzed.

In the laboratory, after the number of galls on a leaf was recorded, each gall was cut off from the leaf at the level of the surface using a blade cutter. Galls were dissected in a petri dish with forceps. When galls were attacked by larvae of *Pipiza* sp. (Diptera: Syrphidae) that prey on the aphids, the galls (1.9%) were removed from the count. One gall usually harbored 1 foundress, and on average, 100 winged mothers. We

dissected 290 galls, but when galls contained only immature larvae, only the number of foundresses per gall was recorded. The total number of winged mothers per gall was counted in 147 galls, for which the dry mass except aphids was weighed with an electric balance (Sartorius BP210D) after the pieces of the dissected gall were dried for 24 h at 60 °C in an oven. Where most second generations in a gall attained adulthood or the fourth (final) stadium, we selected 10 aphids haphazardly from the clone members and dissected them under binoculars using forceps and a fine insect pin in a petri dish with 80% ethanol. The number of male and female embryos that each mother contained in her abdomen was counted. The sex of the embryos was easily determined by the shape and size; female embryos were much larger than male embryos. The mean number of male and female embryos per mother and the clonal sex ratio were calculated for 133 galls; 11 of the 147 galls contained instars that were too young to examine the number of male and female embryos.

In the analysis, we divided the galls into 2 categories: galls with a single foundress and communal galls with 2 or more foundresses. One-foundress galls were used for analyzing sex allocation, but the results from communal galls were subsequently compared with those from one-foundress galls.

Embryos in ovarioles

To understand sex allocation control by *K. japonica*, the number of ovarioles in each winged mother and the number of male and female embryos in each ovariole were counted by dissecting 47 winged mothers collected from 5 galls. Furthermore, to estimate the sex allocation pattern, we measured the area of each male or female embryo based on slide-mounted specimens. The area of slide-mounted male or female

embryos can be used as a reliable index of maternal investment in each sexual (Akimoto et al. 2012). Male and female embryos from each mother were mounted on 1 glass slide by using Hoyer's medium. One day after mounting, the images of mounted sexuals were captured into a computer through a microscope eyepiece camera (Dino-Lite, AM423X Dino-Eye) installed on a binocular microscope (Olympus SZX12). The area of the digitized image for each male or female embryo was measured using the software ImageJ 1.42q after the original images were transformed into binary data (black and white pixels) at a threshold density. For the measurements, 28 male and 55 female embryos from 7 winged mothers were used.

Statistics

For each gall, we obtained the following data: dry gall weight, number of foundresses (the first generation), number of winged mothers (the second generation), number of male and female embryos per mother (the third generation), and total number of galls on the same leaf. The sex ratio was calculated at the individual level for each mother and also estimated at the clonal level based on 10 mothers selected randomly from each gall (Fig. 1). We evaluated the effects of gall weight on the number of aphids in the second or third generation and sex allocation using regression analysis. In some cases, the relationship was described by non-linear regression in a form of $y = a/(1 + b \cdot \exp(c \cdot x))$, where x represents gall weight. The selection of the linear or non-linear regression model was determined based on Akaike's information criterion (AIC). Sex allocation was estimated at the clonal level as the ratio of maternal investment in the entire males from a gall to the total maternal investment. Investment in each male (or female) was assessed from the area of the digitized image of a male (or female)

embryo. Thus, investment in the entire males (or females) was calculated as the mean area of a male (or female) embryo times the total number of males (or females).

We estimated variance components for the number of male or female embryos and the sex ratio per mother by using ANOVA, in which galls were treated as the random effect. In this analysis, the whole variance was divided into the among-gall component and the within-gall component by using the restricted maximum likelihood method (REML).

The effect of foundress number on the number of winged mothers was evaluated by ANCOVA, in which the gall category (one-foundress galls or communal galls) was treated as the independent variable and dry gall weight as the covariate. The effect of dry gall weight on the total number of male and female embryos produced by winged mothers from the gall was also analyzed by ANCOVA after dry gall weight, and the total embryo numbers were log-transformed. In this ANCOVA model, dry gall weight was treated as the covariate and sex as the independent variable. The interaction between the sex and dry gall weight was included in the model to examine whether the slopes for males and females could be regarded as the same or not. Log-transformation was performed to ensure the homoscedasticity of the residuals.

Furthermore, to assess to which extent dry gall weight and the total number of winged mothers per gall affect the average number of female embryos per winged mother or total number of female embryos per gall, we performed multiple regression, in which dry gall weight and the total number of winged mothers per gall were used as independent variables. All statistical analyses were conducted with JMP ver. 9.0.2 and R 2.14.0.

Results

Gall weight and sex allocation

Of 290 dissected galls, 80.3% contained 1 foundress, 16.2% contained 2 foundresses, and 3.5% contained more than 2 foundresses. When all galls were pooled, almost all winged mothers had 4 male embryos (Fig. 2). In contrast, there was a large variation in the number of female embryos that a mother produced (Fig. 2). When the variance in female embryo number was decomposed, the among-gall variance component accounted for 90.6% of the variance, while the within-gall component explained 9.4%. This result suggests that most of the variation in female embryo number is found among galls. On the other hand, for the number of male embryos, the among-gall variance component accounted for only 33.2%, while the within-gall variance component accounted for 66.8%. Furthermore, REML analysis for the individual-level sex ratio showed that 82.3% of that variance was explained by the among-gall variance component, suggesting that the sex ratio also varied mainly among galls. In 1 of the 133 galls examined, all the mothers had entirely female embryos.

When all galls were pooled, the dry weight of a gall had a significantly positive effect on the number of winged mothers produced in the gall (linear regression, $df = 1$, 137, $t = 15.22$, $P < 0.0001$). This result suggests that the larger the gall, the better food resources it provided for the gall inhabitants. When galls were categorized into 2 groups depending on whether they had 1 foundress or more, the results of ANCOVA indicated that communal galls contained more winged mothers than did one-foundatrix galls of the same dry weight (Fig. 3, for the gall category, $df = 1$, $F = 20.4$, $P < 0.0001$; for the interaction between the gall category and gall weight, $df = 1$, $F = 0.99$, $P = 0.321$). This result implies that sex allocation patterns should be analyzed separately

for one-foundress galls and communal galls.

When analysis was limited to one-foundress galls ($n = 108$), the result of regression analysis showed that the dry weight of a gall had a significantly positive effect on the average number of female embryos per mother in the gall (Fig. 4, $df = 1, 105, t = 4.35, P < 0.0001$), but no effect on the average male number per mother ($df = 1, 105, t = 1.05, P = 0.298$). Thus, a good nutritional condition in galls not only increased the number of winged mothers, but also the average number of female embryos that the mothers contained. Multiple regression showed that the average number of female embryos per winged mother was also affected by the total number of winged mothers per gall; this effect was negative, suggesting that higher density of winged mothers per gall reduced the amount of resources per winged mother (multiple regression, $R^2 = 0.264$, for winged mothers, $t = -3.98, P = 0.0001$; for gall weight, $t = 6.11, P < 0.0001$). The total number of male embryos per gall increased with an increasing number of winged mothers per gall, but it was not affected by the dry gall weight (multiple regression, $R^2 = 0.946$, for winged mothers, $t = 33.6, P < 0.0001$; for gall weight, $t = -0.27, P = 0.791$). However, the total number of female embryos per gall was positively affected both by the total number of winged mothers per gall and by the dry gall weight (multiple regression, $R^2 = 0.373$, for winged mothers, $t = 2.23, P = 0.028$; for gall weight, $t = 4.53, P < 0.0001$). On the whole, as the total number of female embryos produced in a gall increased, the total number of male embryos in that gall also increased (Fig. 5, linear regression, $df = 1, 106, t = 5.30, P < 0.0001$).

The clonal sex ratio was 0.548 when averaged over one-foundress galls. The measurements of the area for male and female embryos implied that the cost of producing a female embryo is, on average, 2.09 times that of producing a male embryo. Thus, in *K. japonica*, the clonal sex allocation was calculated to be 0.398 on average,

and it decreased with increasing dry gall weight (Fig. 6); the relationship between gall weight (x) and sex allocation (y) was better described by logistic equation than by linear regression (logistic equation, $y = 0.28/(1 - 1.12*\exp(-75.59*x))$, AIC = -19.42; linear regression, $df = 1, 105, t = -2.85, P = 0.0052, AIC = -18.19$). The decrease in the sex allocation was because the total number of females increased more rapidly with increasing gall weight than did the total male number. The slope of the regression line to gall weight was 0.634 for males and 1.651 for females; ANCOVA indicated that the difference in the slope between the sexes was significant (for the interaction between the sex and dry gall weight, $df = 1, F = 4.73, P = 0.031$).

Exploitative competition and sex allocation

Increasing gall density on a leaf had a negative effect on the performance of individual galls. When all galls were pooled, with an increasing number of galls on a single leaf, the dry weight of a gall decreased (linear regression, $df = 1, 201, t = -5.19, P < 0.0001$), and the number of winged mothers produced in a gall also decreased ($df = 1, 145, t = -2.33, P = 0.0209$); in contrast, the clonal sex allocation increased ($df = 1, 131, t = 7.02, P < 0.0001$).

With the increasing number of foundresses that coexisted in a gall, the dry weight of the gall increased (linear regression, $df = 1, 189, t = 7.99, P < 0.0001$), and the number of winged mothers produced in the gall also increased ($df = 1, 144, t = 13.28, P < 0.0001$). However, the difference in the number of foundresses in a gall did not affect the sex allocation at the gall level (linear regression, $df = 1, 131, t = -1.06, P = 0.293$).

Embryos in ovarioles

Of 47 dissected winged mothers, 87% had 8 ovarioles, and the remaining 13% had 7. Every ovariole was specific to male or female production: there were no ovarioles where both male and female embryos were produced. In every winged mother, 4 ovarioles were utilized for producing female embryos, while the remaining 4 or 3 (13%) ovarioles were for producing male embryos. Of the male-producing ovarioles, 99% contained only 1 male embryo, and the rest contained 2. On the other hand, of the female-producing ovarioles, 69% contained 2 female embryos, 22% contained 1, and 9% contained 3. During dissection, we often found aborted embryos, which appeared to be female embryos.

Discussion

Our results indicate that individual winged mothers produced a constant number of males and a varying number of sexual females depending on the nutritional status of their gall. This result seemingly supports Yamaguchi's hypothesis (1985) that predicts a constant number of males under LMC. This feature has been widely observed among aphid species: *Prociphilus oriens* (Yamaguchi 1985), *Tetraneura nigriabdominalis* (Tanaka 1961), *Kaltenbachiella elsholtriae* (Kurosu and Aoki 1991), *Eriosoma crataegi* (Moran 1993), and *Pemphigus spyrothecae* (Foster and Benton 1992). However, several authors have indicated that the constant male hypothesis could be realized only under limited reproductive conditions (May and Seger 1985; Frank 1987; Kindlmann and Dixon 1989; Stubblefield and Seger 1990). While each mother is developing sexual offspring, she has to know the level of her own reproductive

resources, the number of foundresses, and the average level of reproductive resources of other foundresses in a patch she will join in the future. If these conditions are not satisfied, constant investment in males cannot be expected. In fact, autumnal winged mothers (sexuparae) of eriosomatine aphids produce sexuals in their abdomen before they migrate to a patch on the primary host plant (May and Seger 1985; Kindlmann and Dixon 1989). This observation suggests that the data fit the inappropriate model (West 2009).

For aphid breeding populations, it is most difficult to assess patch size, the number of clones in a patch, because patches are not delimited on the surface of branches and the size is highly variable. In contrast, LMC is most likely to occur because some hundreds males of the same clone search for females in a high-density mass. Stubblefield and Seger's self-knowledge model (1990) assumes a situation where mothers are ignorant of the number and reproductive status of other foundresses in the same patch under LMC. The prediction from the model (model IIa) is that when mothers attempt to optimize their sex allocation in the patch, they produce an increasing number of sons as well as daughters as the level of their reproductive resources increases. In this model situation, winged mothers with a fixed sex allocation will join in a patch with various levels of total maternal investment at a certain probability, so that a mother's sons have to encounter varying intensity of LMC in a patch. Under changing intensity of LMC, mothers would gain by enhancing investment in sons as a bet-hedging. The results of the present study corroborated this prediction when we focused on the sex allocation patterns of clones; that is, each clone, not each mother, produced an increasing number of males with increasing dry gall weight. In addition, we found that as gall weight increased, the total number of sexual females from a gall increased more rapidly than that of males; this simple mechanism led to

more female-biased sex allocation in more fecund clones. These results were also consistent with the prediction from the self-knowledge model. Therefore, the results corroborated the hypothesis that more fecund clones reduce resource allocation to males in order to restrain LMC among clonal males. The observed clonal property of sex allocation would not be detected if we examined only individual sex allocation.

So far, there have been few reports on sex ratios at the clonal level, and our knowledge about the genetic basis of sex allocation patterns is completely lacking. However, a few examples clearly reveal that evaluation of the clone-level sex allocation is necessary for understanding the evolution of sex allocation. In *Masonaphis maxima*, a non-host alternating aphid associated with *Rubus parviflorus*, 2 types of clones with different sex allocation patterns coexist in each patch (Gilbert and Raworth 1998). This aphid species produces sexuals from the third to the fifth generation in northern areas with a limited reproductive season, and the duration of the reproductive season varies depending on the hostplant quality in a patch and weather. Type 1 clones produce excess males in the third generation, but excess sexual females in the fourth generation. By contrast, type 2 clones produce excess sexual females in the third generation, but excess males in the fourth generation. If a patch is examined at any generation, the sex ratio is close to 1:1 because of the coexistence of the 2 clone types (Gilbert and Raworth 1998). These 2 types of clones may represent genetic polymorphism under the operation of frequency-dependent selection, although the genetic basis of the dimorphism is not known. In some species of Adelgidae, clones consist of 2 types of sex expression; one type produces winged mothers that contain entirely female embryos, while the other type produces winged mothers that contain entirely male embryos (Steffan 1970; Sano and Ozaki 2011). This example indicates that in some adelgid species, each clone is either male-like or female-like. For the

aphid *Rhopalosiphum padi* in which sexual and asexual lineages coexist in a population, sex allocation was shown to vary among clones (Rispe et al. 1999); sexual clones produced female-biased broods, while some asexual clones produced only males in addition to parthenogens.

The observation of ovarioles suggests that the constant male rule in eriosomatine aphids may result from developmental constraints imposed on the ovary system. In *K. japonica*, each winged mother usually prepared 4 ovarioles for male production, and each of them contained only a single male embryo (with one exception). Lampel (1968-1969) observed that in an eriosomatine aphid, *Pemphigus spyrothecae*, male embryos started to develop earlier than did female embryos within the abdomen of the larval mother, while female embryos developed rapidly from a later stage, and finally reached a larger size at maturity than male embryos. Our observations on *K. japonica* corroborated Lampel's results and showed that male embryos of *K. japonica* reached the maximal size when their mothers were in the late stage of the third stadium. Male embryos developed simultaneously in their respective ovarioles, whereas the development of female embryos advanced sequentially in each ovariole because 78% of the ovarioles contained 2 or 3 embryos. This finding suggests that the number of male embryos is determined at an early stage of development and is difficult to change during their development, whereas the number of female embryos is likely to change reflecting the maternal nutritional status. Some of the growing female embryos may be aborted during development because of insufficient reproductive resources. The precedence of development of male embryos in specialized ovarioles will readily lead to a constant number of males in a mother. This observation might lead to a notion that *K. japonica* aphids cannot adjust their sex allocation adaptively because of the constraints on the ovaries. However, we can understand that this is not true if

comparing sex allocation among closely related species. Although the production of 4 male embryos per winged mother is fixed at the population level, this constraint is easily changed evolutionarily among closely related species. For example, the winged mother of *Pemphigus spyrothecae* always has 2 male embryos and a various number of female embryos (Foster and Benton 1992). In another eriosomatine species, *Prociphilus oriens*, the number of male embryos is not fixed, ranging from 0 to 4 per winged mother (Akimoto et al. 2012).

The fact that the sex allocation of *K. japonica* is, on average, female-biased can be explained by LMC among clonal males. The sedentary life cycle in *K. japonica* could lead to intense LMC if the number of clones that constitute a patch is small. Similarly, Foster and Benton (1992) showed that in *Pemphigus spyrothecae*, which has a similar non host-alternating life cycle, the average investment in males was 13.5%, and that this low investment in males was due to intense LMC. However, the average sex allocation of *K. japonica* to males (39.8%) was much higher than that in *P. spyrothecae* and furthermore, the sex allocation in a host-alternating eriosomatine, *Prociphilus oriens* (22%–24%) (Akimoto et al. 2012). One possibility for the unexpectedly high level of investment in males may be large patch size. If each patch comprises a number of clones, population sex allocation will approach 50% (Stubblefield and Seger 1990). However, information from the life cycle does not support this possibility. In *K. japonica*, patches are assumed to comprise a small number of clones because mating usually takes place among the offspring of winged mothers that emerge from nearby galls on the same day. In contrast, patches in host alternating species appear to include much more clones because clones from the secondary hosts are well mixed during migration. Furthermore, the possible viscous population structure in *K. japonica* results in high relatedness among clones, which would lead to more female-biased sex

allocation (West 2009). Another possibility may be that in *K. japonica*, LRC among related fundatrices may be intense, as well as competition among clonal males. Our observation and Aoyama et al. (2012) indicate that galls compete with one another for nutritional resources on the same leaf, and that high gall density reduces the fecundity of foundresses. On the other hand, it is possible that galls on the same leaf are induced by related foundresses, i.e., daughters of sexual females derived from the same gall. Thus, in *K. japonica*, due to the limited mobility of the winged mothers, sexuals and foundresses, both LMC and LRC could occur and affect maternal sex allocation (West 2009). Intense competition among related foundresses may have favored enhanced investment in males, and the balance between LMC and LRC may have created the high level of sex allocation.

The available evidence suggests that each clone can adjust its sex allocation in accordance with nutritional conditions and invest more resources in sexual females as the available nutritional resources increase. Thus, the sex allocation pattern of *K. japonica* is condition-dependent. This characteristic is similar to Trivers and Willard's hypothesis (1973) on sex allocation, and a similar result is reported from the gall-forming aphid *Tamalia coweni* (Miller and Aviles 2000). Since *T. coweni* is outbreeding, Trivers and Willard's hypothesis could be applied to the sex allocation of this species. However, the condition-dependence in *K. japonica* and other aphids is realized under LMC (Foster 2002), not because of the improved quality of individual offspring. The main factor influencing the level of nutritional resources available to a clone was interference competition among galls, and higher gall density on a leaf led to greater investment in males in each gall. Although the present method for evaluating sex allocation cannot distinguish genetic and environmental components of the variation, there appears to be a large environmental variance for the sex allocation

patterns.

Another curious feature of *K. japonica* is the homogeneity of the sex ratio among clonal members in a gall; because of this feature, the total number of males from a gall increased linearly with increasing clone size. We suggest that the clonal property of sex allocation detected in the present study prevails among eriosomatine aphids on their secondary host plant. Because autumnal winged mothers cannot fine-tune their sex allocation on the patch on the primary host, natural selection may have acted to create the optimal pattern of sex allocation in clonal colonies on the secondary host. Some studies have documented that large, fecund mothers tend to produce more female-biased broods (Moran 1993; Miller and Aviles 2000; Foster 2002; Dagg and Vidal 2004), while mothers under stressful conditions tend to produce more male-biased broods (Moran 1993). In future studies, it is necessary to determine if this tendency for individual aphids can be detected in the clone-level sex allocation.

In conclusion, under LMC and variable resource conditions, our data on clone-level sex allocation supported Stubblefield and Seger's hypothesis (1990). When we focused on sex allocation patterns in individual mothers, Yamaguchi's hypothesis (1985) seemed likely to be supported. However, this hypothesis ignores the fact that in aphids, natural selection acts on clones, not on individual mothers. We have demonstrated sex allocation patterns for some eriosomatine aphid species (Akimoto and Yamaguchi 2004; Akimoto et al. 2012). For future studies, it is necessary to elucidate evolutionary trends in sex allocation throughout the phylogeny of an aphid group to better understand the evolution of sex allocation in aphids.

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Figure legends

Fig. 1. Diagram for 3 generations in *Kaltenbachiella japonica*. The parenthesis indicates generations in galls. The terminology for generations is consistently used in the text and figures.

Fig. 2. Percentage distribution of the number of male and female embryos that a single mother has in her abdomen. Ten (or rarely less than 10) mothers were randomly selected from each of 133 galls. N, the number of winged mothers examined.

Fig. 3. Relationship between the dry weight of a gall and the total number of 2nd-generation winged mothers in the gall. Regression lines were applied separately to 2 types of galls (galls with 1 foundress and communal galls).

Fig. 4. Relationship between the dry weight of a gall and the mean number of male and female embryos that a winged mother has in her abdomen. The mean number was calculated from 10 (or rarely less than 10) mothers randomly selected from each of 108 galls with 1 foundress.

Fig. 5. Relationship between the total number of males and females produced from a gall with 1 foundress. Total numbers were estimated by multiplying the mean number of male and female embryos per mother by the total number of mothers from the gall

Fig. 6. Relationship between the dry weight of a gall and percentage allocation to males in galls with 1 foundress. Non-linear regression was applied to the plots.

Fig. 1

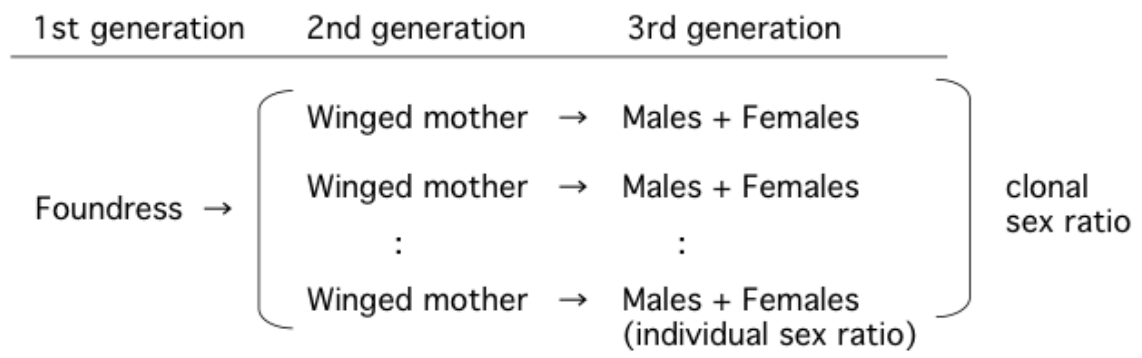


Fig. 2

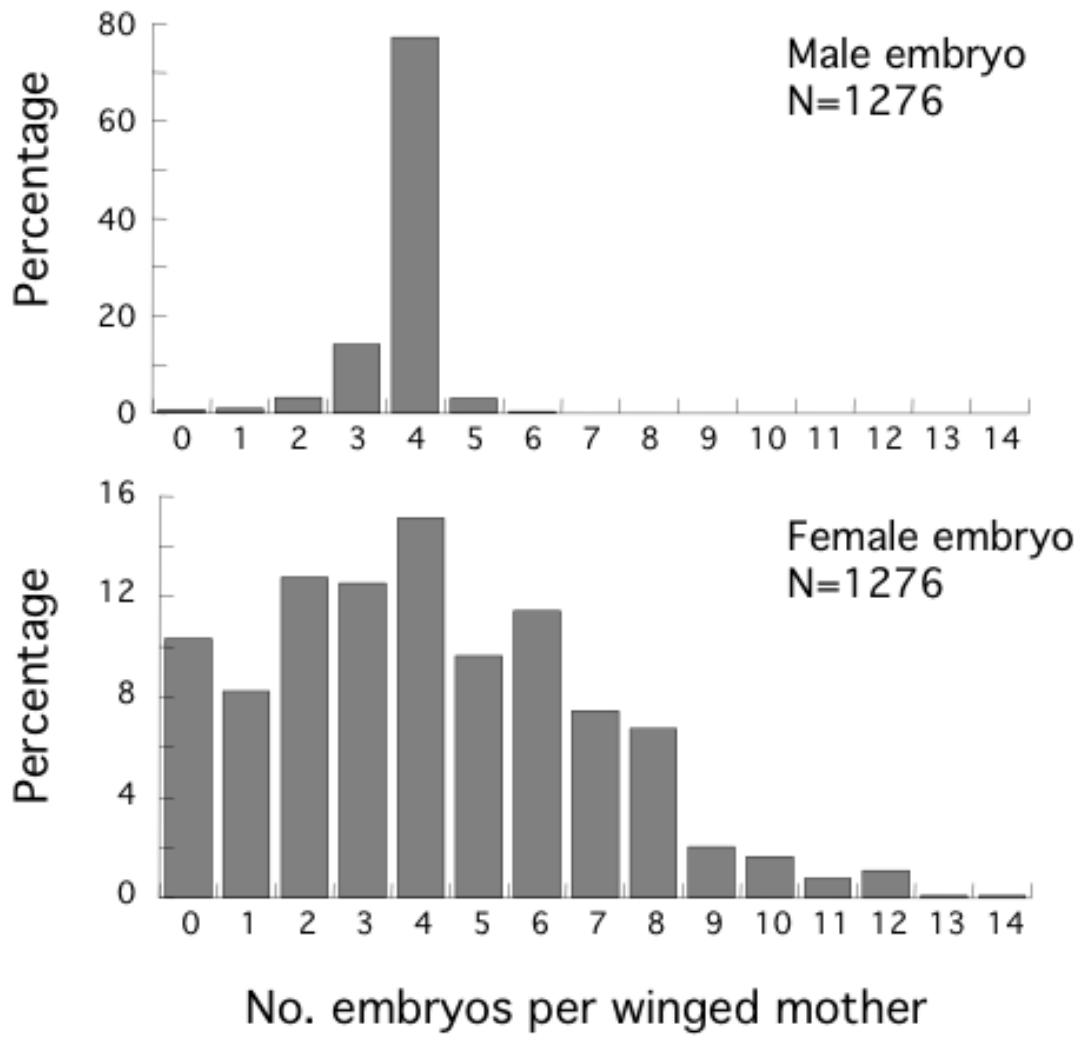


Fig. 3

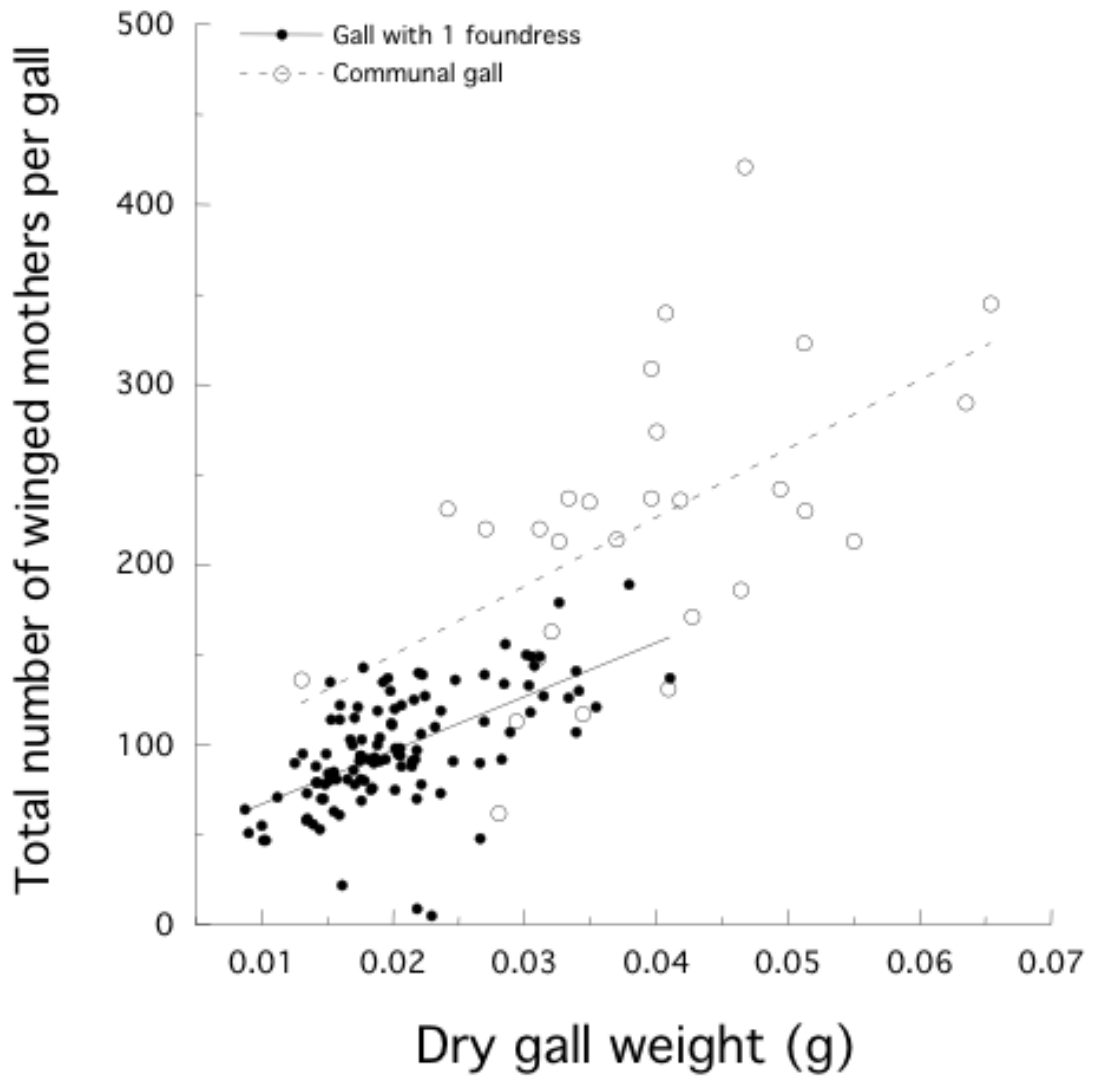


Fig. 4

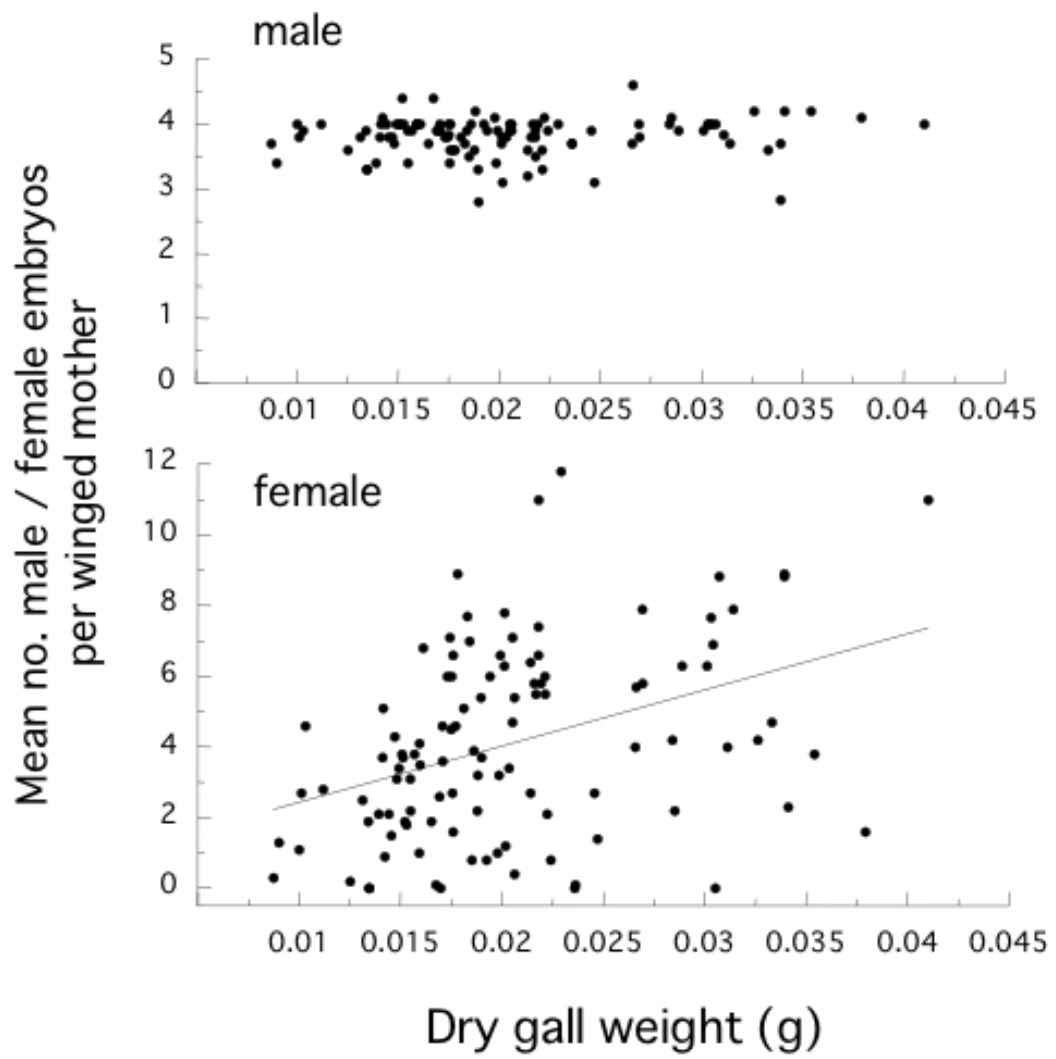


Fig. 5

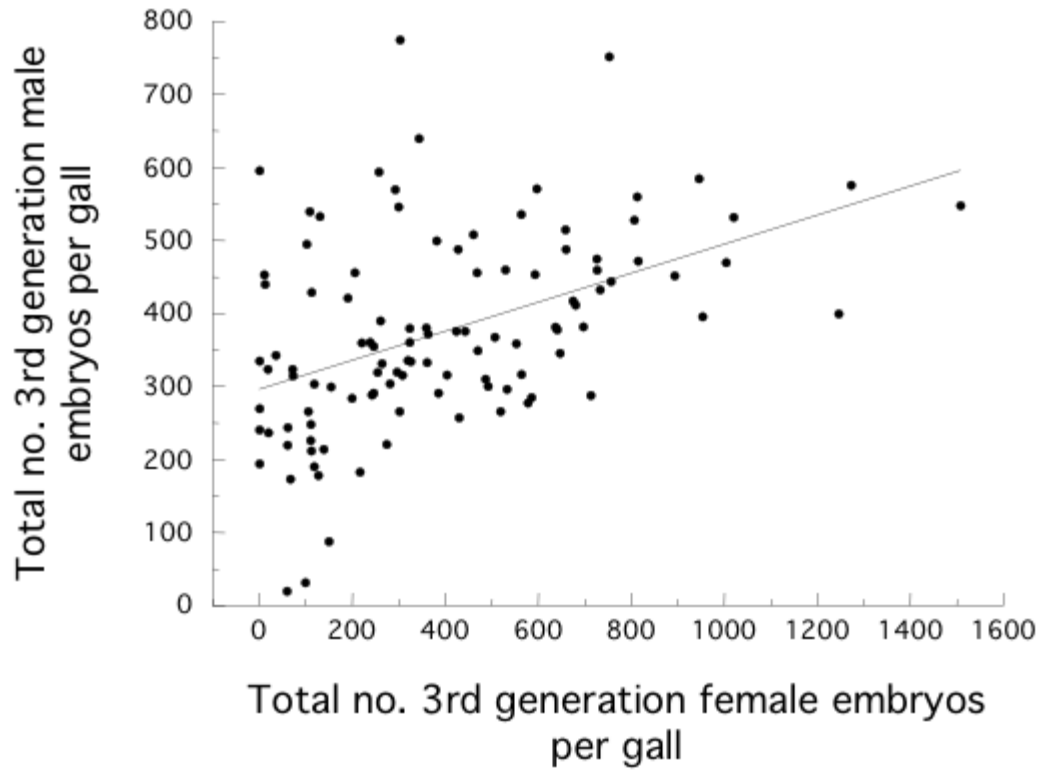


Fig. 6

