



Title	Frequency-dependent selection acting on the widely fluctuating sex ratio of the aphid <i>Prociphilus oriens</i>
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Running title: Frequency-dependent selection

Abstract

Frequency-dependent selection is a fundamental principle of adaptive sex-ratio evolution in all sex ratio theories but has rarely been detected in the wild. Through long-term censuses, we confirmed large fluctuations in the population sex ratio of the aphid *Prociphilus oriens* and detected frequency-dependent selection acting on these fluctuations. Fluctuations in the population sex ratio were partly attributable to climatic factors during the growing season. Climatic factors likely affected the growth conditions of host plants, which in turn led to yearly fluctuations in maternal conditions and sex ratios. In the process of frequency-dependent selection, female proportion higher or lower than ca. 60% was associated with a reduction or increase in female proportion, respectively, the next year. The rearing of aphid clones in the laboratory indicated that mothers of each clone produced an increasing number of females as maternal size increased. However, the mean male number was not related to maternal size, but varied largely among clones. Given genetic variance in the ability to produce males among clones, selection should favor clones that can produce more numerous males in years with a high female proportion. Population-level sex allocation to females was on average 71%–73% for three localities and more female-biased when maternal conditions were better. This tendency was accounted for by the hypothesis of competition among foundresses rather than the hypothesis of local mate competition. We conclude that despite consistent operation of frequency-dependent selection, the sex ratio continues to fluctuate because environmental conditions always push it away from equilibrium.

54 *Keywords:*
55 climate;
56 clonal reproduction;
57 exploitative competition;
58 Eriosomatinae;
59 local mate competition;
60 long-term census;
61 sex allocation;
62 time series.
63

Introduction

Frequency-dependent selection on the proportions of the sexes is a fundamental principle of adaptive sex-ratio evolution, including random mating within a population, local mate competition, and cooperation or competition between relatives (Charnov, 1982; Wrensch & Ebbert, 1993; Hardy, 2002; West, 2009). If the ratio of one sex is lower than equilibrium, females that overproduce offspring of the minority sex can propagate their genes more efficiently, and in the next generation the minority sex increases in proportion. Finally, the sex ratios are hypothesized to reach equilibrium, and thereby a stable, balanced sex ratio is realized because of frequency-dependent selection. The hypothesis of frequency-dependent selection was for the first time proposed by Düsing (1884) and explicitly formulated by Fisher (1930). Subsequently, this hypothesis has been widely accepted and incorporated into mathematical models (Frank 1990; Seger & Stubblefield, 2002). In contrast, testing frequency-dependent selection presents several difficulties. The major difficulty is the high stability of population sex ratios, particularly in mammals and birds (Charnov, 1982; Clutton-Brock, 1986; Clutton-Brock & Lason, 1986). Exceptions to sex ratio stability include those of fishes and reptiles with environmental sex ratio determination (Charnov & Bull, 1989; Korpelainen, 1990; Janzen & Paukstis, 1991) and arthropods with extremely distorted sex ratios because of infection with *Wolbachia* or other cytoplasmic factors (Werren, 1997; Stouthamer *et al.*, 1999; Jiggins *et al.*, 2001; Charlat *et al.*, 2003). Stability of population sex ratios not only makes it difficult to test the operation of frequency-dependent selection, but also only enables us to postulate that random segregation of sex chromosomes in the heterogametic sex is the determinant of 1:1 population sex ratios. It is, however, indispensable to know how frequency-dependent selection functions in wild populations to understand the mechanism underlying sex ratio evolution.

To date, tests of frequency-dependent selection have been conducted by experimentally perturbing the population sex ratio away from 50% (Conover & Voorhees, 1990; Basolo, 1994; Carvalho *et al.*, 1998; Blows *et al.*, 1999). Initial populations with biased sex ratios were established in these studies by using a

temperature-dependent sex determination system in the Atlantic silverside, *Menidia menidia*, a single-locus, three-factor system of sex determination in the platyfish, *Xiphophorus maculatus*, a X-Y meiotic drive system in *Drosophila mediopunctata*, and hybridization between two *Drosophila* species. These studies showed that population sex ratio approached 50% males at different rates of evolution ranging from 3 to 330 generations, depending on the genetic basis of sex ratio determination, thus confirming frequency-dependent selection. However, few studies have documented the presence of frequency-dependent selection on sex ratios in wild populations. One exceptional instance was a drastic increase in the male proportion of the butterfly *Hypolimnys bolina* caused by the rapid spread of a male-rescue allele in populations infected with male-killing *Wolbachia*; that is, in a population with a highly female-biased sex ratio (Charlat *et al.*, 2007). This example suggests that a biological or environmental factor that perturbs the sex ratio away from equilibrium is required to detect frequency-dependent selection in the wild.

Eriosomatine aphids have often been used in sex ratio studies because maternal investment in male and female offspring is readily evaluated through dissection of mothers (Yamaguchi, 1985; Kindlmann & Dixon, 1989; Kurosu & Aoki, 1991; Foster & Benton, 1992; Moran, 1993; Foster, 2002; Akimoto & Yamaguchi, 2004; Akimoto & Murakami, 2012; Akimoto *et al.*, 2012). Aphid mothers parthenogenetically produce male eggs by eliminating one of the X chromosomes, and as such, they can control the sex ratio in the brood. In the Eriosomatinae, winged aphids emerging in autumn parthenogenetically produce male and female embryos in their abdomen and give birth to them on the trunk of the primary host. After birth, without taking food, males and females molt and copulate only using resources provided by their mother. Thus, body mass of offspring is completely determined by maternal investment. Females in this generation produce a single egg, which occupies most of her body. Because female embryos are larger than male embryos (Lampel, 1968-69; Yamaguchi, 1985), maternal investment in both sexes should be evaluated as percentage resource allocation to both sexes when the hypothesis of equal allocation is tested. If production costs for male and female offspring can be assessed appropriately, we could precisely quantify maternal

resource allocation to the two sexes in the brood, in addition to determining the numerical sex ratios of broods and populations (Akimoto *et al.*, 2012).

The pioneer work in the Eriosomatine was undertaken by Yamaguchi (1985), who put forward the constant male hypothesis (Frank, 1987) for *Prociphilus oriens* Mordvilko, 1935 and provided evidence for it by postulating local mate competition (LMC) among males of the same brood. The constant male hypothesis states that given variation in reproductive resources among mothers and LMC, each mother should produce the same number of males and that the remaining resources should be allocated to females (Frank, 1985; Yamaguchi, 1985). Therefore, this hypothesis predicts that mothers with a larger amount of resources should produce more female-biased broods. Later studies revealed exceptions to the constant male hypothesis (Moran, 1993; Akimoto *et al.*, 2012). In eriosomatine species, including *P. oriens*, some fraction of mothers produce all-female broods, which are fertilized by males from other mothers. Nevertheless, Akimoto *et al.* (2012) indicated that there is still a significant tendency for larger *P. oriens* mothers to produce larger broods with a more female-biased sex allocation. This study suggests that *P. oriens* mothers have the potential to adjust sex allocation of their offspring depending on the amount of resources they acquired during development.

Given condition-dependent sex allocation in *P. oriens*, population-level sex allocation can be expected to vary among localities and years, as in other animals with an environmental sex determination system (Conover & Heins, 1987; Charnov & Bull, 1989; Korpelainen, 1990; Janzen, 1994; Ospina-Álvarez & Piferrer, 2008). In fact, a significant difference in the population sex allocation was detected among different localities (Akimoto *et al.*, 2012). Autumnal winged females (mothers) develop on the roots of the secondary host plant, *Abies sachalinensis* (Fig. 1), and their growth condition depends on the conditions of the host plant. We hypothesized that if climatic conditions affected the growth of host trees, then maternal resources, on average, would increase in years when good climate induces vigorous growth of host plants, and vice versa, resulting in yearly fluctuations in maternal resources and the resulting population sex allocation. Fluctuations in population sex allocation from year to year would

provide an opportunity to test frequency-dependent selection in the wild.

To understand selective pressures on and the genetic basis of sex allocation control in *P. oriens*, we have established three research goals: (1) long-term censuses for numerical sex ratios, (2) assessment of female fertility under varying sex ratio conditions, and (3) assessment of clonal sex allocation through rearing of clones. Firstly, we recorded the population sex ratios for 14 consecutive years or more at three localities to evaluate the variation among years and localities. We aimed to understand which factors were responsible for the variation and tested if frequency-dependent selection was acting on the numerical sex ratio. Secondly, we examined the factors that were linked to reproductive failure in females in the breeding populations. Thirdly, we attempted to rear *P. oriens* clones to understand how clones control their sex ratios under variable host conditions. Synthesizing this information, we demonstrated how and why the population sex ratio fluctuates widely in *P. oriens*, and why frequency-dependent selection was detected specifically in *P. oriens*.

Host-alternating aphids reproduce parthenogenetically from spring to autumn, with different female morphs appearing continuously (Fig. 1). This study focused on autumnal winged females and the following sexual generation. To distinguish the two generations, the sexual female is hereafter referred to as the ‘female’, whereas the autumnal winged female is hereafter referred to as the ‘mother’ or sexupara. Males and sexual females are collectively called ‘sexuals’. Some sexuparae produce bisexual broods in their abdomen (Fig. 1), whereas others produce all-female broods (Akimoto *et al.*, 2012), and these were abbreviated as all-F sexuparae.

In the following analyses, we used female proportion in a population as an index of the numerical sex ratio. Our previous study showed that the production cost of one female is, on average, 1.85 times as much as that of one male (Akimoto *et al.*, 2012), so that we calculated a mother's investment in the brood as the number of male embryos plus 1.85 times the number of female embryos. Population-level investment in males and females was, respectively, represented by the sum of the numbers of male embryos and 1.85 times the sum of the numbers of female embryos produced by the sexuparae examined. The population-level allocation ratio to females was defined as population

investment in females divided by the sum of population investment in males and females, i.e., (1.85 times the total female number) / (1.85 times the total female number + the total male number).

Materials and methods

Aphid collection

Autumnal winged females (sexuparae) appear abundantly on calm days in mid and late October, and a large swarm is formed around *Fraxinus mandshurica* trees, the primary host. Sexuparae were collected around *F. mandshurica* trees during flying using an insect net (Akimoto *et al.*, 2012). Aphid species usually include obligatorily parthenogenetic lineages in the population (Simon *et al.*, 2002). However, this species consists only of cyclical parthenogenetic lineages.

Sexuparae were collected in three sites in Hokkaido, northern Japan: (1) the campus of Hokkaido University, Sapporo (43° 4' 12" N, 141° 20' 22" E), (2) Kasuga Cho, Iwamizawa (43° 11' 30" N, 141° 46' 48" E), and (3) Forestry Research Institute, Hokkaido Research Organization, Bibai (43° 17' 3" N, 141° 51' 11" E), where Yamaguchi's study (1985) was conducted. The distance between Sapporo and Iwamizawa was 38.3 km, that between Sapporo and Bibai was 47.6 km, and that between Iwamizawa and Bibai was 11.4 km. Collection was yearly conducted from 2002 to 2015 in all the three localities, and these samples were used for comparison of sex ratios among the localities. In addition, samples collected in Sapporo in 1994 and 2000, samples collected in Iwamizawa in 1998, 2000 and 2001, and samples collected in Bibai from 1981 to 2000 (data of some years are missing) were added to some analyses.

Sexuparae collected on one day of mass flying were used for representing the sex ratio in the locality because a previous study showed no significant differences in sex ratios among collection days in the period of mass flying (Akimoto *et al.*, 2012). Collected sexuparae were preserved in vials of 80% ethanol and then dissected in a Petri dish under a binocular to count the numbers of male and female embryos. In each of

population samples, on average, 167 ($\pm$ 67.8 (SD)) sexuparae were randomly selected, and their abdomen was dissected in 80% ethanol by using two fine insect pins to take out embryos. Embryos of each sex were of almost the same size, and female embryos were larger in size with a wider abdomen. Embryos were readily sexed under a binocular on the basis of their size and shape.

Variance among mothers' investments was decomposed into three variance components; the component among years, that among localities within years, and that within localities, and the percentage contributions of these components were calculated based on the restricted maximum likelihood method (REML) using JMP ver.9.0.2. (SAS Institute Inc., Cary, NC, USA).

Oviposition success in different sex ratios

To explore the relationship between the sex ratio in a breeding population and female oviposition success, we transferred different numbers of virgin males and females in an experimental arena and examined how many females successfully oviposited their single eggs. For this experiment, eight treatments were prepared; 1) 12 males + 2 females (with 52 replicates), 2) 12 males + 4 females (with 25 replicates), 3) 12 males + 6 females (with 8 replicates), 4) 12 males + 12 females (with 25 replicates), 5) 4 males + 12 females (with 25 replicates), 6) 1 male + 12 females (with 25 replicates), 7) 1 male + 43 females (with 14 replicates), and 8) 1 male + 50 females (with 14 replicates). All experiments except 7) were conducted in 2013 and the remaining experiment was prepared in 2015. Immature males and females were collected from the Iwamizawa population. At the evening of the day of sexupara mass flying, paper gauze (Haize gauze, Asahikasei®) that had been placed on the branches of *F. mandshurica* was collected and brought to the laboratory. Newly born sexuals were collected from the paper gauze. At 16°C, 44 h and 51 h, respectively, were needed for males and females to reach sexual maturation. We transferred males and females to the arena before their final molt. The experiments were conducted at 16°C using small styrol containers (30 × 30 × 10 mm) lined with a sheet of dampened filter paper.

Clonal sex ratios

In order to understand clonal sex ratios, we reared *P. oriens* colonies clonally on the twigs of the primary host, *Fraxinus mandshurica* and subsequently on the roots of the secondary host, *Abies sachalinensis*, until the appearance of sexuparae (see Fig. 1). *Fraxinus mandshurica* twigs, ca. 50 cm long, were cut off in early May and hydroponically cultured in plastic bottles (750ml) filled with water at room temperatures in the laboratory. Hatched first instar foundresses, collected from the Iwamizawa population, were transferred onto the cut twigs by using a fine writing brush, and they were allowed to grow to adulthood and reproduce parthenogenetically. To prevent contamination of different clones, we maintained a single foundress that reproduced earliest on the twig by removing others. Foundresses produced about 50 second-generation larvae, which moved to growing leaves and developed into winged adults in late May in the laboratory. Winged adults from the same clone were collected into small styrol containers (30 × 30 × 10 mm), which were lined with a sheet of dampened filter paper. As a stimulant of larviposition, we placed a young shoot of *Abies sachalinensis* in the container. Thirty six to 48 h later, winged adults parthenogenetically produced first instars of the third generation, which were used for the establishment of the clonal colony on the roots of a single *A. sachalinensis* tree.

Prior to the transfer of first instars, young *A. sachalinensis* trees ca. 60 cm tall were transplanted in pots in early May, and they were maintained in a garden of private land in Iwamizawa, Hokkaido (43° 11' N, 141° 46' E). In 2008, 2009 and 2011, we prepared two, 12, and 12 potted trees, respectively. Because *Prociphilus oriens* colonies maintain mutualistic relationships with *Lasius* ants on *A. sachalinensis* roots from early June to mid October, rearing of *P. oriens* colonies requires continuous visits and attendance by the ants. To keep constant visits of *Lasius japonicus* workers, we put diluted honey on the basal stems of *A. sachalinensis* trees twice a day using a dropper. After workers were always observed to forage around the stem bases, first instars that were born in the containers were released to the stem base using forceps. First instars from different clones were separately transferred onto different *A. sachalinensis* trees. However, in 2011 first instars of one clone were transferred onto two trees to understand

the effect of host trees on the sex allocation. We observed that *Lasius japonicus* workers soon approached and tapped first instars with their antennae, and carried them into an ant nest near the stem base with holding them between their mandibles. The release of first instars was conducted each time when they were born in the container. After the release, each potted *A. sachalinensis* tree was covered with a net to prevent other winged adults from alighting on the tree until the appearance of winged adults ended. These trees were kept intact during summer, and again netted from late September on to collect sexuparae emerging from the trees. Sexuparae were daily collected into vials of 80% ethanol. Finally, *P. oriens* colonies on two (100%), seven (58.3%), and seven (58.3%) *A. sachalinensis* trees successfully produced sexuparae in 2008, 2009 and 2011, respectively.

After the collection of sexuparae, the total number was counted for each *A. sachalinensis* tree (colony). Fifty to 68 sexuparae were selected per colony, and each adult was dissected in a droplet of Hoyer's medium on a slide glass by using two fine insect pins. For each sexupara, male and female embryos and one wing were mounted under two cover glasses on a single slide glass. The mounted specimens were then placed at 60°C in an incubator for 3 h. Wing length was measured by using ImageJ version 1.47 (Abràmoff *et al.*, 2004, available from <http://rsbweb.nih.gov/ij/>), and the relationship between maternal wing length and the numbers of male and female embryos was examined. We also evaluated the numerical sex ratio per mother and a mother's total investment as mentioned above.

Statistics

The basic statistics for the numbers of males and females produced by a mother were calculated for the Bibai population, in which samples were collected for the longest term.

To test if the observed yearly fluctuations in female proportions tend to return to a specific value (i.e., they show stationarity) or not, we first conducted the unit root test for the time-series data in the three localities using the Augmented Dickey-Fuller test (adf.test) in R version 3.2.1 (R Core Team, 2015). If stationarity is rejected by the test

(i.e., fluctuations follow a random walk process), then the data will be composed of dependent samples, possibly producing a seeming trend with time. This result violates the assumption of correlation and regression analyses. Thus, in comparisons between different time-series data, we took difference between neighboring years before we calculate the correlation between two time-series data.

We tested if fluctuations in the proportions of females and all-F sexuparae and maternal investment are correlated with environmental variables or not by using stepwise multiple regression analysis. In the model, localities (three sites) and six metrological parameters during the growing season (precipitation in July, August and September, and mean temperature in July, August and September) at each locality were specified as explanatory variables. The explanatory variables were selected in a stepwise manner (the backward method) based on the Bayesian information criterion (BIC) using JMP ver.9.0.2. Metrological data at three localities (Bibai, Iwamizawa, and Sapporo) were obtained from the database of Japan Metrological Agency (<http://www.data.jma.go.jp/obd/stats/etrn/index.php>). In addition, we tested to what extent female proportion in a year affected changes in that proportion the next year (proportion in year i – proportion in year $i-1$) by using ANCOVA. In the model, change in female proportion in year i was treated as the response variable, while female proportion in year $i-1$, locality, and the interaction between them were treated as explanatory variables.

In mating experiments, the number of females that successfully produced fertilized eggs relative to the number of females that failed was analyzed using logistic regression, in which female proportion in experimental arenas and its square were treated as explanatory variables. The square term was added to the model because preliminary observations showed that female proportions in arenas had non-linear effects on female fertility. The glm function in R (R Core Team, 2015) was used for the analysis with a binomial error structure.

In rearing experiments, factors affecting the occurrence of all-F sexuparae were tested with logistic regression. In the model, clones and maternal wing length were specified as explanatory variables, and whether a brood is bisexual or includes females

only was specified as the binary response variable. Subsequently, we evaluated factors affecting the number of females a mother produced using ANCOVA, in which clones, maternal wing length, and the interaction between them were treated as explanatory variables. The same analysis was also applied to the number of males per mother.

To know whether the numerical sex ratio per mother varies significantly among colonies or not, we analyzed the numbers of female embryos relative to male embryos by using the glm function with a binomial error structure. Difference in the sex ratio between any two of the 16 colonies (120 pairwise combinations) was tested, and the sequential Bonferroni adjustment (Rice, 1989) was applied to the calculated *P* values to keep the table-wide *P* value constant at 0.05.

Results

Time series analysis

During the period from 1981 to 2015, sexuparae in the Bibai population produced zero to four male embryos (mean = 2.89 ± 1.56 (SD)) and a variable number of female embryos ranging from zero to more than 11 (mean = 4.16 ± 2.57 ; Table 1). Approximately 60% of sexuparae produced four males, but 17% produced all females. The proportion of sexuparae producing four males and that of sexuparae producing all females in each year were negatively correlated throughout the period (Spearman's $r = -0.865$, $n = 20$, $P < 0.0001$), and thus we focused only on the proportion of all-F sexuparae in later analyses.

Fluctuations in female proportions were shown for the Sapporo, Iwamizawa, and Bibai populations (Fig. 2), along with fluctuations in the proportions of all-F sexuparae. Female proportions fluctuated widely from 46.5% to 67.8% (mean = $57.2\% \pm 6.0$) in Sapporo, from 49.1% to 76.0% (mean = $59.5\% \pm 8.5$) in Iwamizawa, and from 46.4% to 84.3% (mean = $59.4\% \pm 11.0$ (SD)) in Bibai. In initial study years (in 1981 (data not shown) and 1993) in Bibai, the proportion of all-F sexuparae was less than 3% but gradually increased and started to fluctuate widely after 2002, with a maximum of 52.3% in 2009. Population-level resource allocation was highly female-biased; allocation to females was, on average, 72.3% throughout the populations (71.3% in

Sapporo, 73.1% in Iwamizawa, and 72.5% in Bibai).

Augmented Dickey-Fuller tests indicated that the hypothesis of random walk was not rejected for the 2002–2015 data of the Iwamizawa and Bibai populations, suggesting that the time-series of female proportions are not stationary (for Iwamizawa, Dickey-Fuller = -1.96, $P = 0.587$; for Bibai, Dickey-Fuller = -1.70, $P = 0.687$). In contrast, stationarity was confirmed for the Sapporo population (Dickey-Fuller = -4.07, $P = 0.021$). This result implies that stabilizing forces act more intensely on fluctuations in the Sapporo population. However, since fluctuations in the Iwamizawa and Bibai populations are non-stationary, correlation analyses involving these fluctuations do not satisfy the condition of independence of data. Thus, the following correlation analyses were performed by taking differences between neighboring years.

The fluctuations of female proportions in the Bibai population were significantly correlated with those in the Sapporo and Iwamizawa populations (for Sapporo, $n = 13$, $r = 0.561$, $P = 0.0462$; for Iwamizawa, $n = 13$, $r = 0.620$, $P = 0.0239$). However, no significant correlation was detected between the Sapporo and Iwamizawa populations ($n = 13$, $r = 0.166$, $P = 0.587$). Fluctuations in the proportions of all-F sexuparae were significantly correlated between all combinations of the Sapporo, Iwamizawa, and Bibai populations (for Sapporo-Iwamizawa, $n = 13$, $r = 0.641$, $P = 0.0182$; for Sapporo-Bibai, $n = 13$, $r = 0.675$, $P = 0.0114$; for Iwamizawa-Bibai, $n = 13$, $r = 0.784$, $P = 0.0015$). This result implies effects of regional factors common to the three localities on these fluctuations. In every population, fluctuations in female proportions were highly and positively correlated with those in all-F sexupara proportions (Fig. 2; for Sapporo, $n = 13$, $r = 0.850$, $P = 0.0002$; for Iwamizawa, $n = 15$, $r = 0.879$, $P < 0.0001$; for Bibai, $n = 13$, $r = 0.947$, $P < 0.0001$). Similarly, fluctuations in female proportions were positively correlated with those in the mean maternal investment (for Sapporo, $n = 13$, $r = 0.917$, $P < 0.0001$; for Iwamizawa, $n = 15$, $r = 0.854$, $P < 0.0001$; for Bibai, $n = 13$, $r = 0.787$, $P < 0.0014$). Of the variance among mothers' investments, 5.9% was accounted for by the among-year component, 8.0% was by the among-locality component, and 86.1% was by the within-locality component.

Stepwise multiple regression analysis indicated that climatic factors during the

growing season were highly correlated with the proportions of females and all-F sexuparae and mean maternal investment; these response variables decreased with increasing precipitation in August and increasing temperature in September, while the mean maternal investment increased with increasing temperature in August (Table 2).

Female proportion in a year had a significantly negative effect on changes in that proportion the next year (ANCOVA, $R^2 = 0.55$; $df = 1,35$, $F = 36.65$, $P < 0.0001$; Fig. 3); a value higher than ca. 60% in a year led to a large reduction in female proportion the next year, whereas a value $<$ ca. 60% resulted in an increase in female proportion the next year. However, neither of locality and the interaction between female proportion and locality affected changes in female proportion in the next year (for locality, $df = 2,35$, $F = 1.13$, $P = 0.334$; for the interaction, $df = 2,35$, $F = 1.52$, $P = 0.232$).

The previous analysis indicated the significant effect of climatic factors on female proportion. Thus, we recalculated the effect of frequency-dependent selection by adding precipitation in August and temperature in September to the model (Table 3). Analysis based on this model (ANCOVA, $R^2 = 0.70$) resulted in lower AIC (288.0) than that in the previous model (302.6) and revealed significant effects of precipitation in August and female proportion in the previous year (Table 3).

Oviposition success in different sex ratios

The proportion of females that successfully produced fertilized eggs exhibited a unimodal pattern to female proportion (Fig. 4). The oviposition success rate was positively correlated with female proportion ($z = 8.15$, $P < 0.0001$) and negatively with the square of female proportion ($z = -9.61$, $P < 0.0001$); the oviposition success rate reached a maximum when male number was one third of female number (Fig. 4). In arenas where more than 40 females were present per male ($n = 28$), males fertilized a median of 18 females and a maximum of 35 females. In arenas where males are more numerous than females, females often died during molting or oviposition due to interference from males.

Clonal sex ratios

From 2008 to 2011, we successfully reared 15 aphid clones for which the relationships among maternal size, maternal investment and female proportions were evaluated. All clones but one produced all-F sexuparae, although their proportion varied largely among clones (Fig. 5), ranging from 79.0% in clone C to 0 % in clone LY5. This result suggests that all-F sexuparae did not come from specific clones. The proportion of all-F sexuparae in a clone was highly correlated with female proportion among the clones ($r = 0.882$, $n = 15$, $P < 0.0001$). In 2008 and 2009, most clones produced all-F sexuparae at the highest proportion, but in 2011 four of six clones produced sexuparae producing a 4-male brood at the highest proportion (Fig. 5). This tendency was similar to the patterns of male numbers in the Iwamizawa population, from which the clones were derived; the proportion of sexuparae producing a 4-male brood increased abruptly in 2011 (Fig. 5). Logistic regression showed that the proportion of all-F sexuparae varied significantly among clones ($df = 14$, $\chi^2 = 233.9$, $P < 0.0001$), but was not correlated with maternal wing length ($df = 1$, $\chi^2 = 0.60$, $P = 0.437$).

The total number of sexuparae emerging from each colony was not correlated with the clonal sex ratio (Table 4, $r = -0.060$, $n = 15$, $P = 0.832$) or the mean wing length of sexuparae ($r = -0.152$, $n = 15$, $P = 0.589$). Among clones, maternal investment averaged over clone members was positively correlated with the averaged wing length of mothers ($r = 0.864$, $n = 15$, $P < 0.0001$). ANCOVA indicated that female number per mother varied significantly among clones ($df = 14, 752$, $F = 21.9$, $P < 0.0001$) and was correlated with maternal wing length ($df = 1, 752$, $F = 447.4$, $P < 0.0001$); the interaction between clones and maternal wing length also had a significant effect on female number ($df = 14, 752$, $F = 6.0$, $P < 0.0001$). In addition, the mean female number per mother was significantly correlated with the mean maternal investment ($r = 0.630$, $n = 15$, $P < 0.0001$). Male number per mother varied significantly among clones ($df = 14, 752$, $F = 26.0$, $P < 0.0001$), but was not correlated with maternal wing length ($df = 1, 752$, $F = 0.65$, $P = 0.420$) or affected by the interaction between them ($df = 14, 752$, $F = 1.44$, $P = 0.129$).

The numerical sex ratio was compared among 16 colonies on *A. sachalinensis* trees, two of which (LY5 and LY6) were derived from the same clone (Table 4). No

significant difference was found in sex ratio between LY5 and LY6 colonies.

Discussion

Because of the widely held belief that the sex ratio is balanced and maintained at equilibrium, long-term studies on sex ratio variation have rarely been conducted. Our long-term censuses on the sex ratio of *P. oriens* revealed that female proportion fluctuated widely from year to year. It should be noted that female proportion in a year affected the proportion the next year in all of the three populations; when female proportion is higher or lower than ca. 60% in a year, it tended to decrease or increase, respectively, the next year. Therefore, this analysis shows that frequency-dependent selection has been acting on the sex ratio. This study is the first report of frequency-dependent selection acting over a long period in the wild. The present study also indicates that the amount of maternal investment, which is highly correlated with maternal size, fluctuated from year to year. Among-year variation in maternal investment accounted for 5.9 % of the entire variance. We observed higher female proportions in years when mothers had a larger amount of reproductive investment on average. These findings imply that the growth conditions of host plants probably influenced the reproductive investment of mothers, who could adjust sex allocation according to the resources they acquired during development.

The finding that fluctuation patterns of female proportion and all-F sexupara proportion were similar among the three localities suggests that these proportions are influenced by large-scale regional factors such as climate. A statistical model incorporating metrological variables verified this hypothesis; high precipitation in August and high temperature in September were strongly linked to decreases in mean maternal investment, female proportion, and all-F sexupara proportion. In Hokkaido, higher precipitation in August typically comes from heavy rainfall caused by typhoons. We speculate that heavy rain may have hindered the aphids' feeding activities on the host roots and detrimentally affected maternal resources. In addition, high temperature in September may have negatively affected the physiological conditions of *Abies sachalinensis*, which is adapted to cold climates. In contrast, high temperature in August

is linked to longer sunshine duration, which may have promoted the photosynthesis and growth of host trees and resultantly led to a larger amount of maternal investment. In aphids, environmental impacts on a generation are reported to influence the phenotypes of later generations through the telescoping of generations (Mondor *et al.*, 2004; Podjasek *et al.*, 2005; Keiser & Mondor, 2013; Tegelaar *et al.*, 2013). Thus, climatic impacts could affect the population sex ratio through transgenerational effects.

Frequency-dependent selection should have acted as the stabilizing force for fluctuations in female proportion, leading to an equilibrium of ca. 60%. However, there was no evidence that fluctuations in female proportions are attenuating with time. We detected large residual variance in the regression analysis of changes in female proportion (Fig. 3), and 38% of the residual variance was attributed to yearly changes in climatic factors. The results of the ANCOVA indicated that both frequency-dependent selection and climatic factors accounted for the fluctuations of female proportions. This result suggests that climatic changes caused wide fluctuations in female proportions, which were in turn adjusted by frequency-dependent selection. Therefore, in *P. oriens* whose sex allocation is affected environmentally, fluctuations in female proportions would not decline despite consistent operation of frequency-dependent selection.

The arena experiments with different sex ratios revealed that males enjoyed reproductive advantages when they were minority. Males have the ability to inseminate as many as 18 females, and this high ability may be an adaptation to high female proportions. In this situation, natural selection would act positively on clones that can produce more males. Male proportion was approximately 40% on average in the wild, implying that male-male competition should be moderate. However, when male proportion became higher than 50%, females less successfully produced fertilized eggs because of persistent male courtship behavior, which often disturbed female molting and oviposition. In 17% of 53 population samples, male proportion exceeded 50%. With increasing proportion of males, clones producing higher proportion of females would be favored by selection to mitigate interference from males and male-male competition. Therefore, the mating experiment suggests that females adapt to breeding populations with a high female proportion (ca. 75%), where they can produce fertilized eggs most

safely.

The fact that sex ratio is condition-dependent has been reported in some aphid species (Yamaguchi, 1985; Foster & Benton, 1992; Moran, 1993; Miller & Aviles, 2000; Wool & Sulami, 2001; Foster, 2002; Dagg & Vidal, 2004; Akimoto & Murakami, 2012; Akimoto *et al.*, 2012), and this characteristic has almost always been explained in terms of LMC. The present study showed that population-level allocation to females was 71%–73% for the three localities, implying that strong LMC might have occurred in the breeding populations. However, whether the LMC hypothesis is the only rationale for female-biased sex allocation in aphids was questioned by Akimoto *et al.* (2012), who pointed out that female-biased and condition-dependent sex allocation could have evolved from aphid life cycles.

Aphid males appear only in one generation and mate with sexual females to produce fertilized eggs. Only females hatch from fertilized eggs, founding new clonal lineages (so called foundresses). In Eriosomatine aphids, body mass of a first-instar foundress is solely determined by the sexupara's investment in each female because every female, without feeding, deposits one large egg, from which a first-instar foundress hatches (Heie, 1980; Fig. 1). Thus, maternal investment in females is equivalent to investment in her offspring foundresses (granddaughters). In aphids, a sexupara's granddaughters (foundresses) and their clonal offspring could compete with unrelated females over limited resources on the same host plant. In this situation, if a mother's extra investment in females can positively contribute to the outcome of competition among foundresses, then the mother could gain higher marginal genetic returns from investment in females than in males.

Several sex allocation theories indicate that if the function that relates maternal investment with genetic returns is different between the two sexes, then equal allocation is not expected (Trivers & Willard, 1973; Charnov, 1979; Maynard Smith, 1980; Frank, 1990; Seger & Stubblefield, 2002). Theory predicts that if a mother's fitness returns from investment in sons (x) and daughters ($1 - x$) are respectively represented as x^a and $(1 - x)^b$, then the ESS sex allocation to sons (x') is calculated as $x' = a / (a + b)$ (Seger & Stubblefield, 2002). Therefore, in case of $b > a$, that is, if investment in females results

in higher marginal returns, the sex ratio is expected to be female-biased. In this theoretical framework, Trivers & Willard (1973) predicted that if mothers have different amounts of reproductive resources, mothers should exhibit different sex allocation patterns, with more fecund mothers allocating more toward the sex with the greater rate of reproductive returns. These non-linear models (Frank, 1990) can be applied to aphid life cycles, in which competition could occur among foundresses. Given competition among unrelated foundresses, a mother's sex allocation would be distorted by selection so as to enhance the reproductive output of her offspring foundresses. In contrast, obvious fighting has not been observed among males in this study or by Foster & Benton (1992). Therefore, more intensive competition between unrelated foundresses than male-male competition may have led to the evolution of female-biased sex allocation. This prediction can be applied to other organisms with cyclic parthenogenesis if the breeding population consists mainly of unrelated individuals.

Competition in the foundress generation can arise in two ways, physical fighting and exploitative competition. Examples of combat are known from gall-forming aphids, in which first-instar foundresses fight with one another over the ownership of a suitable galling site or an incipient gall (Whitham, 1979; Aoki & Makino, 1982; Akimoto, 1988; Akimoto & Yamaguchi, 1997). During fighting, larger body size is overwhelmingly advantageous (Whitham, 1979; Aoki & Makino, 1982; Akimoto & Yamaguchi, 1997). In *Tetraneura sorini*, foundresses are reportedly subject to directional selection for larger body size in all populations examined (Muramatsu & Akimoto, 2016). Therefore, where unrelated foundresses frequently fight, mothers can gain higher fitness returns by investing more resources in individual females, thereby producing larger foundresses. In *T. sorini*, highly female-biased sex allocation has been reported (Akimoto & Yamaguchi, 2004).

In species whose foundresses do not fight, including *P. oriens*, investment of more resources in females is also advantageous for mothers. If a mother can produce more females than do others, she can have more granddaughters (foundresses) in spring, thereby acquiring a head-start advantage over the succeeding exploitative competition among clones. If a larger number of offspring foundresses can occupy more new leaves,

then they will overwhelm unrelated foundresses more easily on the same plant. In addition, Akimoto (2006) indicated that in *P. oriens*, greater investment in a female (and her egg) resulted in the foundress with disproportionately higher fecundity. This is because the allometry of foundress gonad mass to egg size is positive with an allometry coefficient of 1.5. In aphids, exploitative competition among conspecifics or heterospecific clones is reportedly severe (Moran & Whitham, 1990; Inbar *et al.*, 1995; Fuller *et al.*, 1999). Furthermore, the head-start effects have been corroborated by the prevalence of asexual clones in areas where sexual and asexual lineages coexist (Rispe & Pierre, 1998; Rispe *et al.*, 1998; Dedryver *et al.*, 2001). When sexual clones hatch from overwintered eggs in spring, asexual clones have already begun to reproduce on the same plant during mild winters, occupying suitable feeding sites. Thus, in spite of the advantage of genetic diversity, sexual clones are overwhelmed by asexual clones during mild winters.

The foundress competition hypothesis for female-biased sex ratios can be applied to almost all aphid species and predicts that more fecund mothers should produce a more female-biased sex ratio (Trivers & Willard, 1973). This tendency has been detected in aphid species whose life cycles are unlikely to lead to LMC (Ward & Wellings, 1994; Miller & Aviles, 2000). In addition to the possibility of foundress competition, local mate competition among sibling males or local resource competition among sibling foundresses (Dagg & Vidal, 2004) might also arise in *P. oriens*. The relative magnitude of the three kinds of competition will determine sex allocation. However, in host-alternating aphids, because a large number (usually thousands) of winged mothers aggregate on a single primary-host tree, we postulate that competition among unrelated foundresses should be much stronger than competition among sibling foundresses or among sibling males. When mothers' density is low and the mobility of males and foundresses is limited, local mate competition and local resource competition will play an important role. However, it should be noted that effects of these two factors are likely to be canceled out in aphids with limited mobility (West, 2009; Akimoto & Murakami, 2012).

The appearance of all-F sexuparae cannot be accounted for by the LMC

hypothesis (Yamaguchi, 1985) or its modified version (Stubblefield & Seger, 1990), which predicts that mothers produce a fixed number or an increasing number of males as maternal size increases. The LMC hypothesis predicts the optimal sex allocation for mothers joining a local breeding population. However, in aphids, we have to determine the optimal sex allocation strategy of clones rather than individual mothers because the clone is a unit of natural selection (Moran, 1993; Akimoto & Murakami, 2012). The main finding of the rearing experiments was that almost all clones produced two types of sexuparae: those producing an all-female brood and those producing a bisexual brood (mainly including 4 males). We found that *P. oriens* clones exhibited a wide variety of sex ratio expression, with clones producing a male-biased sex ratio, a female-biased sex ratio, or an intermediate ratio (Table 4, Fig. 5). A large variation in sex ratio was detected among clones or mothers in other aphids (*Masonaphis maxima*, Gilbert & Raworth, 1998; *Rhopalosiphum padi*, Rispe *et al.*, 1999; *Tamalia coweni*, Miller & Aviles, 2000; Adelgidae, Steffan, 1970; Sano & Ozaki, 2011). We hypothesize that all-F sexuparae has evolved from sexuparae producing a bisexual brood because of advantages in clone competition; namely, the former type can produce more females efficiently. In the eriosomatine *Kaltenbachella japonica*, almost all clones produce only sexuparae with bisexual broods, whereas one clone was observed to produce only all-F sexuparae (Akimoto & Murakami, 2012). Dimorphism in *P. oriens* sexuparae may have evolved from the ancestral situation found in *K. japonica*.

Analysis of sex-ratio fluctuations indicated that favorable environmental conditions led to increases in maternal investment and all-F sexupara proportion, which in turn increased female proportion. This finding implies that when climatic conditions are favorable, each clone increases the proportion of all-F sexuparae relative to sexuparae producing bisexual broods. The developmental mechanism that determines the two types of sexuparae in a clone remains unknown. The rearing experiment showed that sexupara body size was not the determinant of the dimorphism in each clone. Apart from this environmental effect on the production of all-F sexuparae, individual mothers in every clone had the ability to increase female numbers in response to increasing resources. This condition-dependent female production is probably another mechanism

for clones to be advantageous in clonal competition. Therefore, in *P. oriens*, there are two mechanisms (clonal and individual) to increase female number, and as such, female proportion is likely to increase rapidly under favorable environments.

The fact that male number per mother was not affected by maternal resources but varied largely among clones suggests that male production is less affected by environmental conditions. In the experiment in which the members of the same clone were reared on different host trees (LY5 and LY6), we found that the numerical sex ratios of the two colonies were highly male-biased and did not vary significantly between the colonies. Although detailed genetic experiments were not conducted, there may be a genetic basis for the male-producing ability of clones. Given genetic variation in male-producing ability, frequency-dependent selection can act on this variation.

In conclusion, the present study demonstrated the operation of frequency-dependent selection and a large fluctuation in the sex ratio under the operation of frequency-dependent selection. *Prociphilus oriens* clones can produce a more female-biased sex ratio when they grow in good host conditions, and thus, the population sex ratio fluctuates in response to yearly environmental variation. For organisms without the environmental sex-ratio determination system, it may be difficult to detect frequency-dependent selection because of stable sex ratios. We conclude that although frequency-dependent selection acts consistently, sex ratios continue to fluctuate because environmental conditions drive the sex ratio away from equilibrium.

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827

Figure legends

Figure 1 Life cycle of *Prociphilus oriens*. This species alternates host plants seasonally between *Fraxinus mandshurica* and *Abies sachalinensis*. In autumn, sexuparae (winged females) appear from the roots of *A. sachalinensis* and migrate to the trunks of *F. mandshurica*. Sexuparae have matured male and female embryos in their abdomen. The proportions of female embryos in broods and local populations are investigated. Sexuparae usually produce bisexual broods in their abdomen as shown here but sometimes produce all-female broods (abbreviated as all-F sexuparae). Males (or male embryos) and sexual females (or female embryos) are shown in green and orange, respectively. Each female has a single egg (shown in yellow).

Figure 2 Yearly fluctuations in the proportions of females and sexuparae producing all females (all-F sexuparae) in Bibai, Iwamizawa, and Sapporo. Data of 2002 to 2015 are used for comparison of the fluctuation patterns among the three localities.

Figure 3 Relationship between the proportion of females in a year (x) and changes in female proportion the next year (y). Data from three populations were pooled. The regression line is represented as $y = 55.90 - 0.948 x$.

Figure 4 Relationship of the percentage of females that successfully produced fertilized eggs to the proportion of females in arenas. The horizontal axis is represented on the scale of $\log(\text{female number}/\text{male number})$. Mean percentage oviposition success $\pm$ SE is indicated. The relationship of percentage oviposition success (y) to female ratio, f/m (x) is represented by the equation, $y = 8.33 x - 7.71 x^2 - 1.11$.

Figure 5 Percentage distribution of male number per mother in each clone and in the Iwamizawa population in 2008, 2009, and 2011.

856

857 Appendix S1 Numbers of male and female embryos each sexupara produced in her
858 abdomen. Collection localities and years are indicated.

859

860 Appendix S2 Relationship between climatic factors in a year and total maternal investment
861 and the proportions of females and all-F sexuparae in a population. Total precipitation and
862 mean temperature in July, August, and September are indicated.

Table 1 Number of sexuparae (autumnal winged females) who produced certain numbers of male and female embryos in their abdomen. Data are from 3102 sexuparae collected in Bibai from 1981 to 2015.

No. of males	No. of females												%
	0	1	2	3	4	5	6	7	8	9	10	11≤	
0	0	1	17	30	62	34	81	65	149	38	42	15	17.2
1	0	10	20	18	36	23	27	12	20	2	1	0	5.4
2	11	14	30	25	60	24	28	14	17	3	2	0	7.4
3	24	25	55	50	79	30	34	19	18	4	1	3	11.0
4	172	166	356	263	442	119	161	56	64	6	7	17	59.0

Table 2 Meteorological effects on the proportions of females and [all-F sexuparae](#) and the mean maternal investment. Stepwise multiple regression analysis was applied to these response variables by using eight explanatory variables (two locality variables and six meteorological variables at each locality). Variables indicated as '—' were not adopted into the model by the backward method. * the partial regression coefficient.

Variable	Proportion of females			Proportion of all-F sexuparae			Mean maternal investment		
	Coeff.*	<i>F</i>	<i>P</i>	Coeff.*	<i>F</i>	<i>P</i>	Coeff.*	<i>F</i>	<i>P</i>
Locality (Sapporo vs others)	—	—	—	—	—	—	-0.4048	3.85	0.0555
Locality (Iwamizawa vs Bibai)	—	—	—	—	—	—	—	—	—
Precipitation, July	—	—	—	—	—	—	—	—	—
Precipitation, August	-0.0456	15.95	0.0002	-0.0671	21.32	< 0.0001	-0.0055	6.94	0.0113
Precipitation, September	—	—	—	—	—	—	—	—	—
Temperature, July	—	—	—	—	—	—	—	—	—
Temperature, August	—	—	—	—	—	—	0.5039	9.14	0.0040
Temperature, September	-2.1405	8.73	0.0048	-2.287	6.16	0.0165	-0.6838	15.55	0.0003

Table 3 ANCOVA for changes in the proportion of females in local populations (female proportion in year i – female proportion in year $i-1$).

Explanatory variable	df	Coefficient	F	P
locality	2	—	0.626	0.5409
% females in the previous year	1	-1.004	62.17	<0.0001
precipitation in August	1	-0.075	14.47	0.0002
temperature in September	1	-1.751	3.56	0.0675

Table 4 Numbers of sexuparae and their male and female offspring produced by colonies reared on *Abies sachalinensis* trees. The proportion of females in a colony and the mean wing length of sexuparae were also indicated. Different letters following % females show significant difference at a 5% significance level after the sequential Bonferroni adjustment. All colonies came from different clones except LY5 and LY6, which belonged to the same clone.

Colony	Year	No. sexuparae produced	Mean no. males per mother	Mean no. females per mother	% females in a colony	Mean wing length (mm) $\pm$ SD
J	2008	86	1.84	1.84	50.00 b	3.29 $\pm$ 0.271
L	2008	194	1.27	1.48	53.85 bc	2.95 $\pm$ 0.185
A	2009	459	0.59	2.59	81.37 ef	3.37 $\pm$ 0.318
B	2009	90	0.78	2.27	74.32 def	3.24 $\pm$ 0.213
C	2009	1628	0.42	2.61	86.17 f	3.24 $\pm$ 0.229
D	2009	215	1.34	2.33	63.40 bcd	3.26 $\pm$ 0.318
E	2009	564	0.58	2.25	79.64 ef	3.05 $\pm$ 0.251
G	2009	131	1.76	4.27	70.78 cde	3.58 $\pm$ 0.178
H	2009	1021	1.36	3.39	71.38 cde	3.45 $\pm$ 0.220
LY2	2011	692	0.64	2.82	81.50 ef	3.29 $\pm$ 0.234
LY4	2011	758	2.94	0.68	18.78 a	3.26 $\pm$ 0.162
LY5	2011	705	3.40	0.72	17.48 a	3.19 $\pm$ 0.303
LY6*	2011	345	2.26	0.44	16.30 a	3.02 $\pm$ 0.243
LY8	2011	1128	3.04	0.82	21.24 a	3.37 $\pm$ 0.255
LY9	2011	1142	1.02	1.04	50.49 b	3.01 $\pm$ 0.233
LY12	2011	187	2.80	3.00	51.72 b	3.59 $\pm$ 0.229

* LY6 was excluded from the analysis of clonal differences.

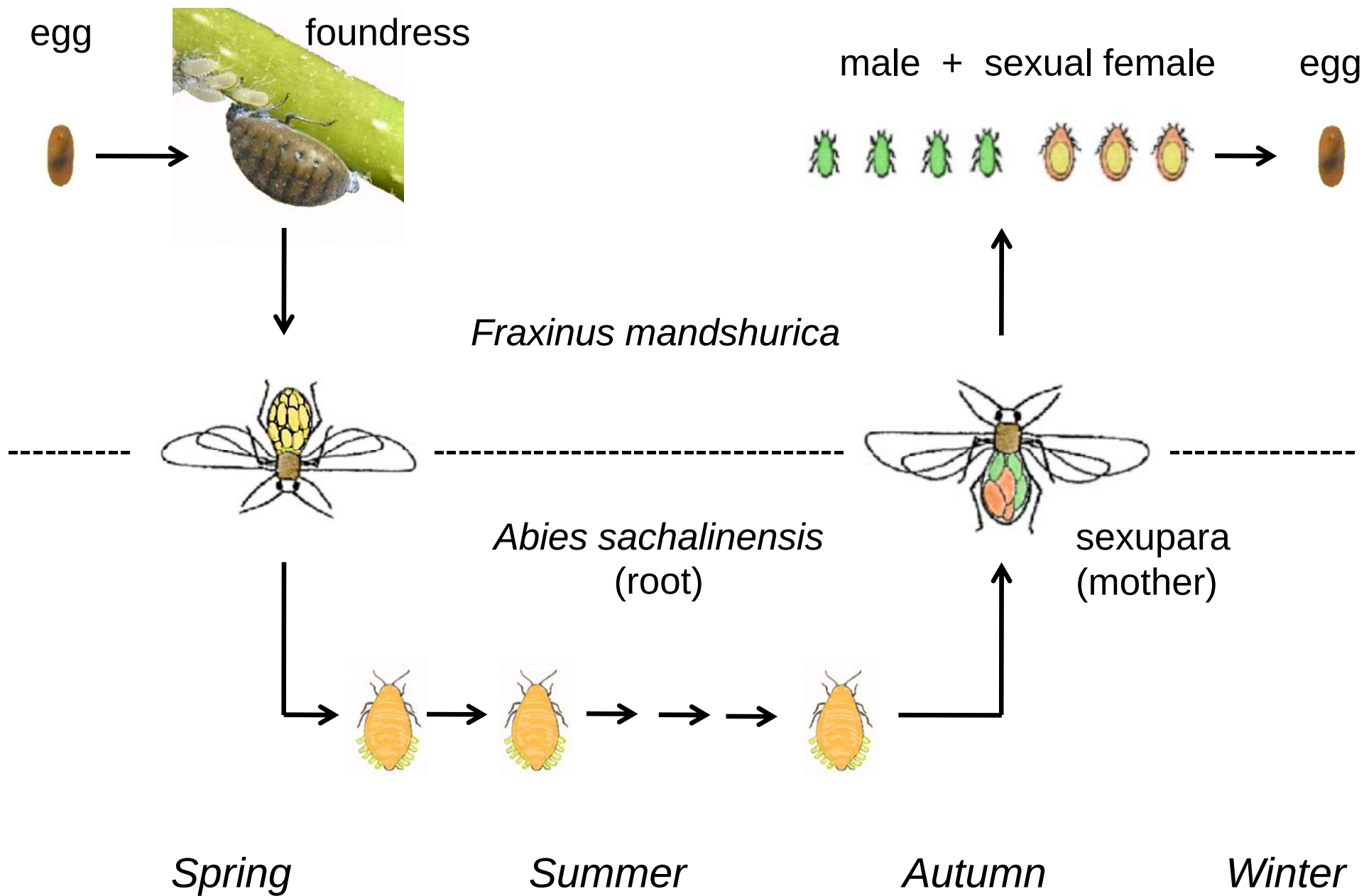
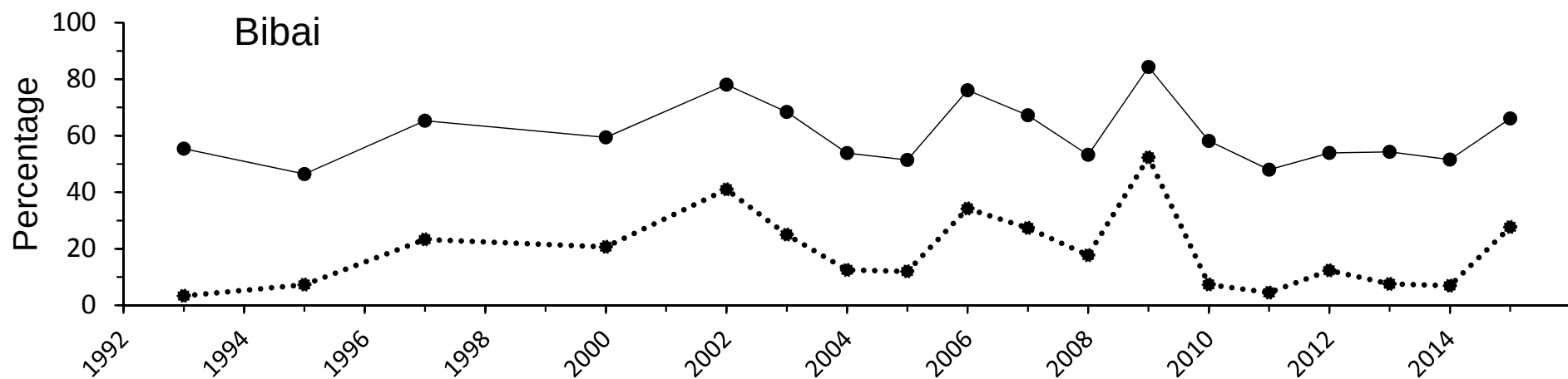
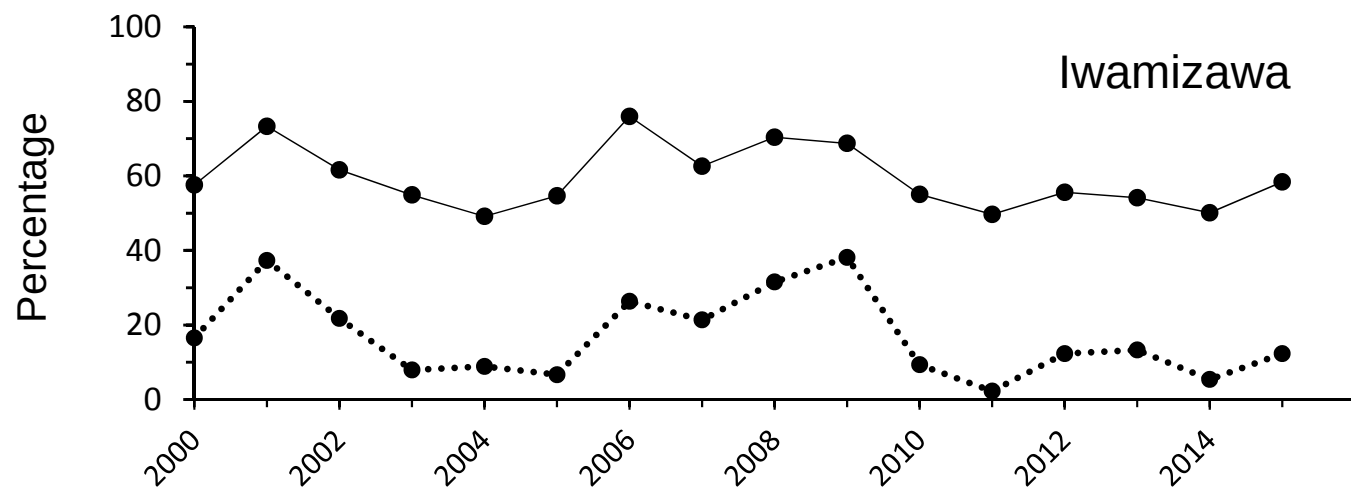
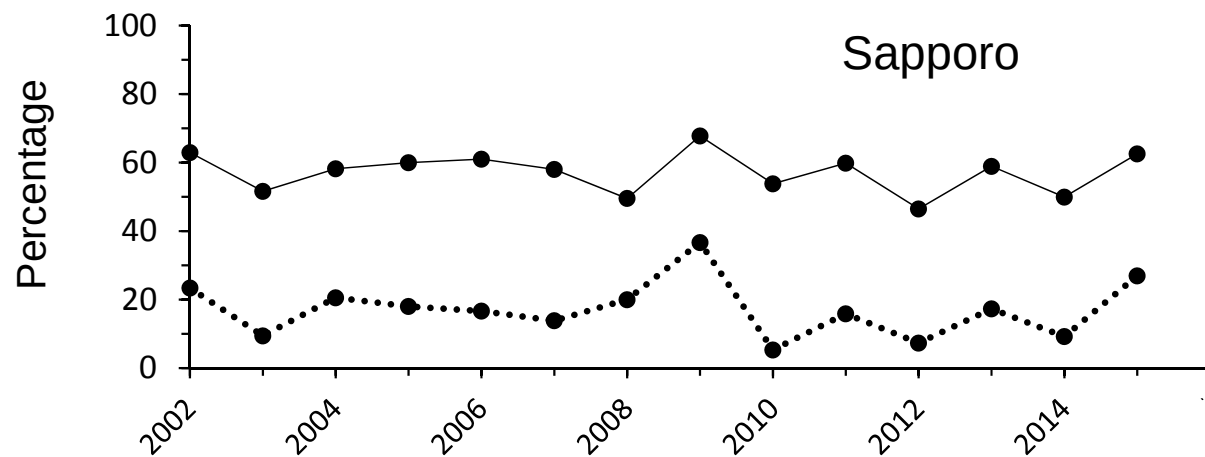


Fig. 2

- % females
-● % sexuparae
producing a
all-female brood



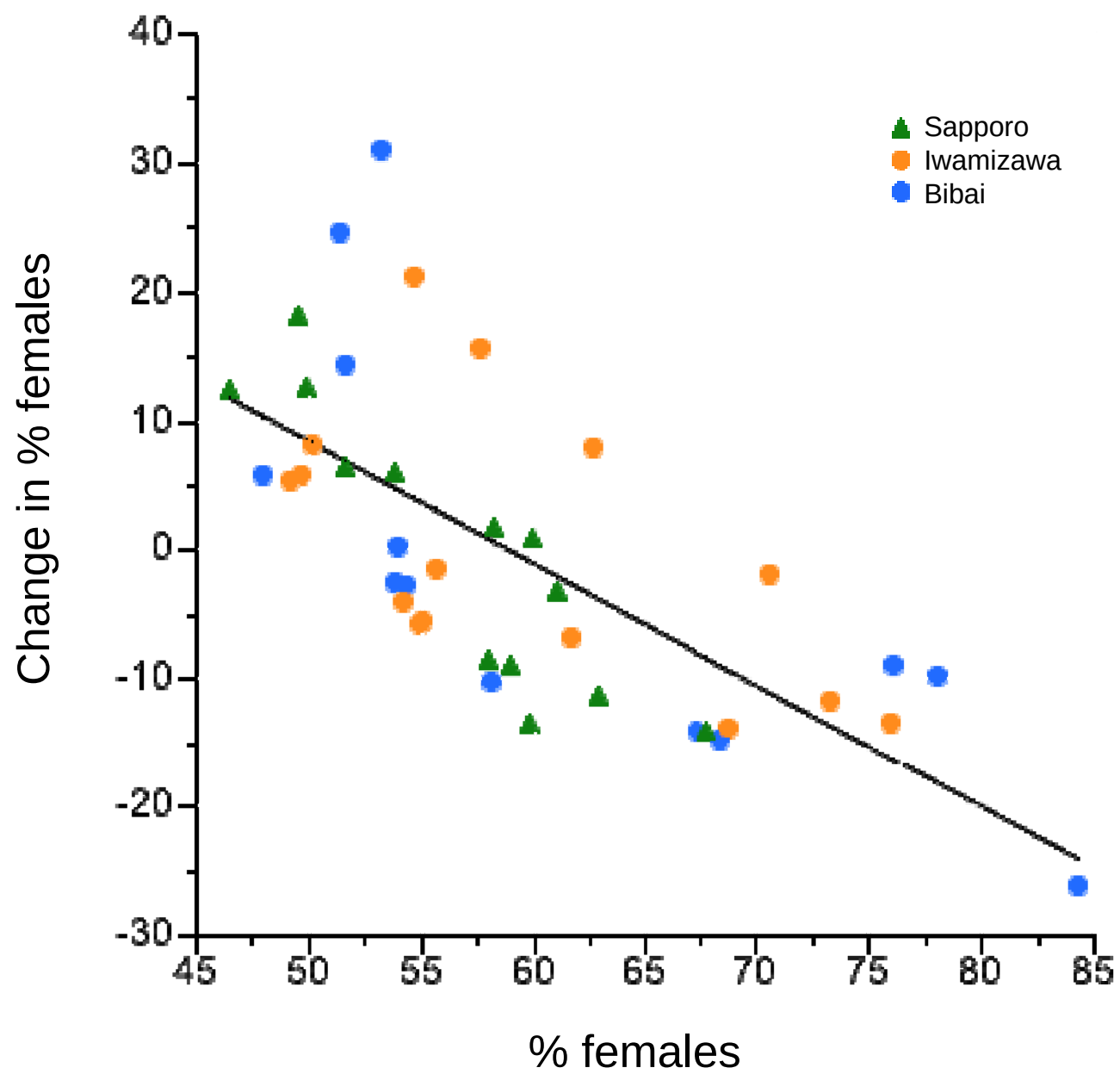


Fig.3

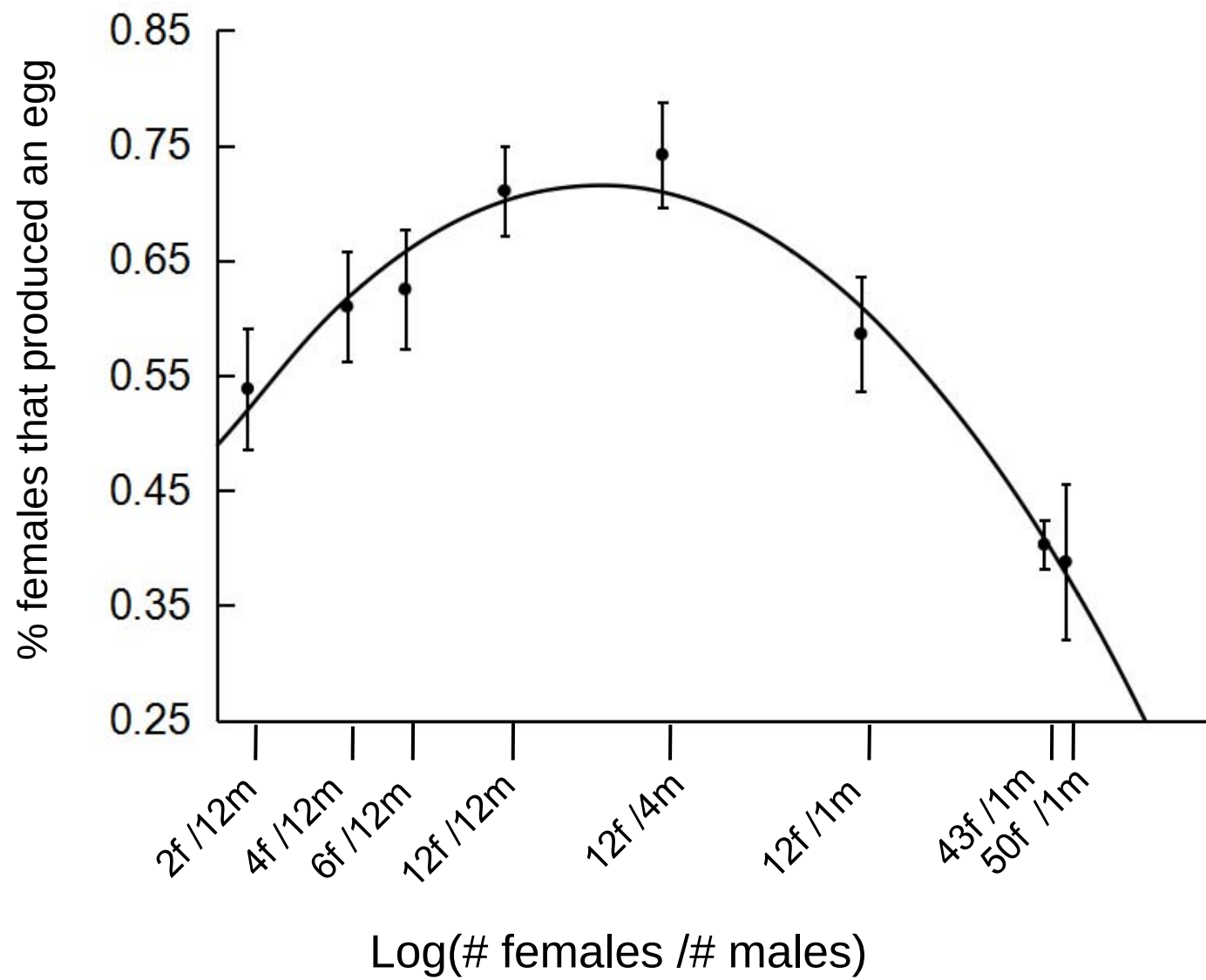
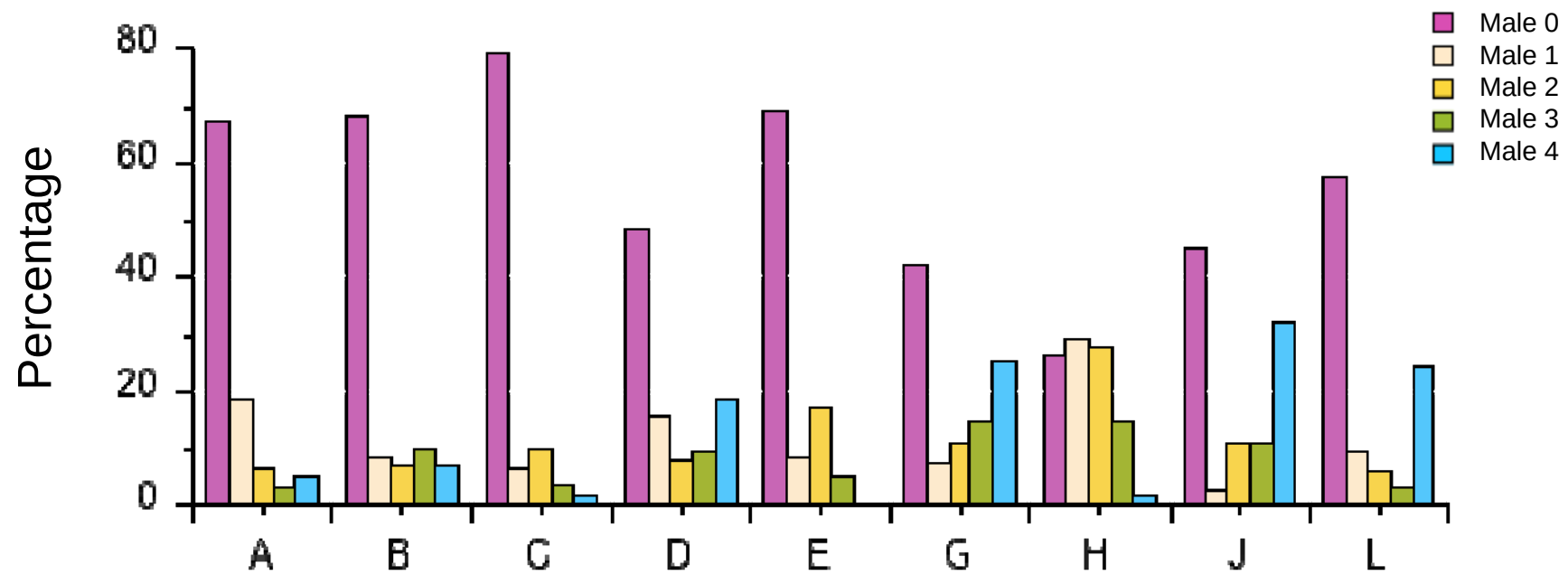
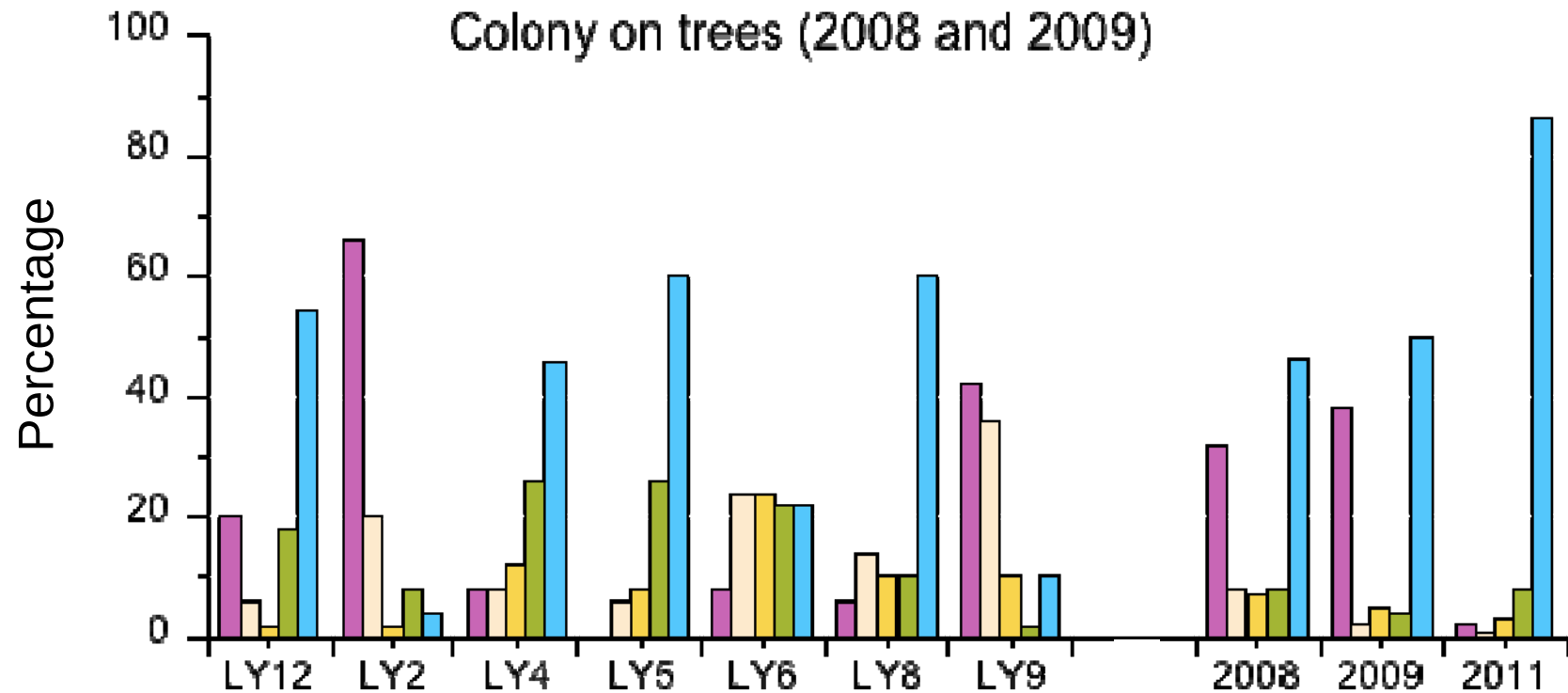


Fig.4



Colony on trees (2008 and 2009)



Colony on trees (2011)

Field