Persistence of a *Wolbachia*-driven sex ratio bias in an island population of *Eurema* butterflies

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Abstract

Prevalence of maternally inherited sex-ratio-distorting endosymbionts such as *Wolbachia* may have profound effects on reproduction and behaviour and can also select for nuclear suppressors in insect populations. In the butterfly *Eurema mandarina*, a *Wolbachia* strain *w*Fem induces female drive, wherein all the offspring develop as female without any sibling lethality. We monitored an island population of *E. mandarina*, which comprises *w*Fem-positive females, *w*Fem-negative females and *w*Fem-negative males, from 2005 to 2017. Despite some fluctuations, *w*Fem appeared to be maintained for at least 12 years at a high frequency in the population, resulting in a female-biased population with a considerable number of virgin females. Because there was no evidence of suppressors against sex ratio distortion, the mechanism acting against increase/fixation of *w*Fem infection, which would otherwise lead to the population extinction, would be either the constant immigration of *w*Fem-negative females or the fitness cost of *w*Fem. Suggestively, the wing size was slightly smaller in *w*Fem-positive females than in *w*Fem-negative females. Additionally, the discrepancies observed between sex ratios of captive individuals and sex ratios deduced from *w*Fem infection frequencies among females can be explained by a plastic behavioural change of males and females in response to the shift of sex ratios.

Keywords: butterfly, female drive, *Eurema mandarina*, population sex ratio, *Wolbachia*

1. Introduction

Arthropods are often influenced by selfish microbes, such as *Wolbachia*, which are maternally transmitted and can distort the sex ratio toward females [1–3]. It is predicted that the prevalence of sex ratio distorters can lead to the extinction of the host population [4], unless genetic suppressors evolve in the host. To date, the existence of host genetic suppressors against sex ratio distorters has been described in various arthropods such as fruit flies, ladybird beetles, butterflies, moths, lacewings and woodlice [5,6], and the spread of suppressors have been documented in the two systems, i.e., butterfly *Hypolimnas bolina* (Lepidoptera; Nymphalidae) [7–10] and the lacewing *Mallada desjardinsi* (Neuroptera; Chrysopidae) [11,12].

In the Samoan island population of *H. bolina*, a strongly female-biased sex ratio—less than 1% male—was first reported in the early twentieth century [13–15]. On the same island, the similarly skewed sex ratio observed again in 2000 was found to be due to the *Wolbachia*-induced male killing [16]. Five years later, the same team found that the sex ratio was near parity on the island [8]. Crossing experiments revealed that this was due to the prevalence of a host suppressor against male killing [7]. Similarly, the spread of suppressors against *Spiroplasma*-induced male killing occurred in less than five years in a population of *M. desjardinsi* in Chiba, central Japan [11,12]. The occurrence of rapid spread of host nuclear suppressors in two independent systems suggests that such events—an arms race regarding the sex ratio between the cytoplasmic and nuclear genomes—may be quite common in insects.

Here, we examined the sex ratio dynamics in an island population (Tanegashima Island, Japan) of the pierid butterfly *Eurema mandarina* (Lepidoptera; Pieridae), wherein all-female-producing females and normal females coexist. In *E. mandarina*, cytoplasmic-incompatibility-inducing *Wolbachia* (*w*CI) is fixed in most of the populations in Japan, including on Tanegashima Island [17,18]. Females singly infected with *w*CI (referred to as C females) produce offspring at a 1:1 sex ratio [17,19]. However, females infected with another strain of *Wolbachia*, *w*Fem, in addition to *w*CI (referred to as CF females), produce all- or nearly all-female offspring [19–22].

It was recently revealed that, like the majority of lepidopteran insects, C

71 females have the WZ karyotype and C males have the ZZ karyotype. In contrast, CF
72 females have the Z0 karyotype [23,24]. By mating with C males, CF females produce
73 embryos that are all Z0, whose Z chromosomes are paternally derived (i.e.,
74 *Wolbachia*-induced disruption of maternal inheritance of the Z chromosome). In the
75 presence of *w*Fem, Z0 individuals are determined as female (i.e., *Wolbachia*-induced
76 feminization), which would otherwise be determined as male in the absence of
77 *Wolbachia* [24]. The remarkable difference between this phenotype and male killing is
78 that the removal of *Wolbachia* results in all-male offspring in this phenotype [20,24] but
79 both male and female offspring in other systems where male killing occurs. Therefore,
80 in CF females, cytoplasmic elements such as mitochondria cannot be transmitted to
81 subsequent generations in the absence of *Wolbachia*—a state of evolutionary addiction
82 to *Wolbachia*. This is congruent with the finding that CF females and C females on
83 Tanegashima Island have distinct mitochondrial haplotypes despite the absence of
84 nuclear DNA differentiation [25]. From a population genetics perspective, this
85 phenomenon can be regarded as female drive [6] because all of the offspring harbour
86 *w*Fem and its associated cytoplasm without any selective sibling lethality.
87 Assuming that equal numbers of offspring are produced by CF females and C
88 females, CF females are expected to produce twice as much daughters as C females. If
89 this is the case, CF females would dominate the population and may lead to the
90 population going extinct because the number of males would become too small to
91 sustain the population. Alternatively, nuclear suppressors against female drive, if they
92 arise, may rapidly increase in the population. With these possible scenarios in mind, we
93 monitored the island population of *E. mandarina* for 12 years.
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96 # 2. Materials and methods

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98 ### (a) Collection of butterflies

99 On Tanegashima Island (Kagoshima Prefecture, Japan), we patrolled the pavement and
100 made efforts to collect all of the *E. mandarina* adults that we encountered. Males were
101 stored at −30°C until DNA extraction. After allowing females to lay eggs in the

laboratory for other experiments [21,24], bursa copulatrixes and Malpighian tubules were dissected out from them and the remains were stored at −30°C until DNA extraction.

(b) Dissection of spermatophores

Following Konagaya and Watanabe [26], bursa copulatrixes were carefully opened under a dissecting microscope and the number of spermatophores inside was recorded.

(c) Measurement of wing size

For adult females, the distance between the tip of the forewing and the base of the forewing jointed with the thorax was measured.

(d) DNA extraction and PCR

DNA was extracted from either legs or thoraxes using DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan). Infection with *Wolbachia* of either *w*CI or *w*Fem strain was identified by specific PCR detection targeting the *wsp* gene [19,21,22,27]. Specifically, *w*CI was detected by using the *Wolbachia*-specific forward primer wsp81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') [28] and *w*CI-specific reverse primer WHecFem1 (5'-ACTAACGTTTTTGTTTAG-3') [19], which amplify a 232-bp DNA fragment and *w*Fem was detected by using the *w*Fem-specific forward primer WHecFem2 (5'-TTACTCACAATTGGCTAAAGAT-3') [19] and *Wolbachia*-specific reverse primer wsp691R (5'-AAAAATTAAACGCTACTCCA-3') [28], which amplify a 398-bp DNA fragment.

(e) Statistical analyses

Analyses of binary data (i.e., virgin or mated females) were performed with generalized linear mixed models (GLMM) using binomial error distribution. Analyses on mating frequencies (i.e., number of spermatophores per female) were performed with GLMM using Poisson error distribution. Random effects of different visits were included in the models. GLMM were fitted using Laplace approximation. All of these statistical analyses were performed using the function glmer of the program package lme4 using the software R version 3.4.4 [29].

3. Results and discussion

(a) CF females consistently produce all or nearly all-female offspring

As was partly shown previously [21,22,25], all but one of the 36 CF females collected on Tanegashima Island produced only daughters (1024 females and no males for 35 broods and 7 females and 1 male for 1 brood; electronic supplementary material, table S1), while all of the 17 C females produced sons and daughters (218 females and 227 males for the recorded 14 broods; electronic supplementary material, table S1). These results confirm that suppressors against female drive are absent or very low in frequency, if present. It is also likely that there are no sex ratio distorters other than *w*Fem in the population.

(b) Female-biased population sex ratio is associated with a high frequency of all-female-producing CF females

In total, 668 adults were captured on Tanegashima Island, Japan from 2005 to 2017 (through 13 visits), which included 523 females and 145 males. Among 13 visits, sex ratios varied (37.9%–100% female; median = 86.5%; electronic supplementary table S2). Out of 495 females diagnosed with *Wolbachia* infection, 495 (100%) were positive for *w*CI and 415 (83.8%) were also positive for *w*Fem (i.e., 80 C females and 415 CF females; electronic supplementary material, table S2).

The proportions of CF females among females (P_{CF}) varied among the 13 visits (54.2%–92.5%; median = 84.4%; electronic supplementary material, table S2). By assuming that (1) equal numbers of C females and males existed in the population and (2) behavioural activity did not differ between C females and CF females, the proportion of females (P_{female}) can be deduced from P_{CF} as

$$P_{female,\ deduced} = \{P_{CF} + (1 - P_{CF})\} / \{P_{CF} + 2(1 - P_{CF})\}$$
$$= 1 / (2 - P_{CF})$$

For the 13 visits, these deduced sex ratios ($P_{female, deduced}$) were compared with the observed sex ratios (i.e., $P_{female, observed}$; proportions of females among captive individuals) (figure 1*a*). These values fit well, except for four visits with the least biased sex ratios (3rd, 6th, 10th and 11th visits; significantly different only on the 10th and 11th visits) and three visits with extremely female-biased sex ratios (1st, 2nd and 13th visits; significantly different only on the 2nd visit). We speculate that these discrepancies are due to the temporary change of male behaviour in response to fluctuation of sex ratio. When males are not extremely rare, males are more conspicuous and easily recognised by investigators than females because males actively search for females one after another despite frequent rejections by females. This is also a typical situation in *E. mandarina* populations without CF females, where the actual sex ratio is 1:1 (observed male to female ratios were 1.5–17 in seven populations; median: 3.22; figure 1*b*). A male-biased sex ratio of captive individuals has also been reported for other butterfly species [30–32]. However, males become less conspicuous under extremely female-biased conditions with a high proportion of virgin females because males would have high rates of mating success. In such extreme conditions, males may become less conspicuous than females; in such a case, females may actively search for males. In her visit in 2005, SN described that females were actively flying, possibly searching for males and that such behaviour is unusual in other populations of *E. mandarina*.

This behavioural change would thus be plastic; indeed, turnovers of observed and deduced sex ratios were observed multiple times (figure 1*a*). However, female-biased conditions, if lasting for long enough, may select for a genetic trait of males with less active mating behaviour or of females with more active mating behaviour. In the butterfly, *Acrea encedon* (Lepidoptera; Nymphalidae), high prevalence of male-killing *Wolbachia* is considered to have resulted in sex-role reversal in mating behaviour [33]. There might already exist some slight genetic changes in mating behaviour in the Tanegashima Island population compared with other populations without *w*Fem.

It is unclear when CF females invaded Tanegashima Island. According to the butterfly inventory recorded from the 1960s, 16 females and 24 males in total were observed in 1960–1978 and 17 females and 16 males in total were observed in 2002–2007 (no significant difference between the two periods by chi-squared test;

electronic supplementary material, table S3). Importantly, SN noted in her graduate thesis that one of the samples collected from Tanegashima Island in 1998 was PCR-positive for *w*Fem. Although we have no idea whether *w*Fem was present in 1960–1978, it is likely therefore that *w*Fem was already present and spread in 2002–2004, before the start of the present study.

(c) Scarcity of males leads to a high proportion of virgin females

The proportion of virgin females is abnormally high on Tanegashima Island (12.2%–82.6%; median = 41.5%) compared with that of other populations without CF females (0%–4.76%; median = 0%) (table 1). Overall, the proportion of virgin females was significantly correlated with the sex ratio ($p = 0.0124$ by GLMM), but they were discrete depending on the season (figure 2*a*). Significant correlations between the sex ratio and the virginity were observed for females collected in June ($p = 0.00554$ by GLMM) and for females collected in September ($p = 0.0102$ by GLMM) (figure 2*a*). Similarly, the number of spermatophores per female was significantly correlated with sex ratio ($p = 0.000724$), but they were discrete depending on the season (figure 2*b*). Significant correlations between sex ratio and number of spermatophores were observed for females collected in June ($p = 0.0379$ by GLMM) and for females collected in September ($p = 0.0179$ by GLMM) (figure 2*b*). Taken together, it is likely that the *Wolbachia*-induced scarcity of males resulted in a low mating frequency on Tanegashima Island. The higher mating tendency in late summer (September) than in early summer (June) may be explained by the higher activity of males due to the elevated temperature. The larger population size in late summer than in early summer may also increase the chance of encounters between males and females regardless of the sex ratio.

In *H. bolina*, *Wolbachia*-induced female bias leads to an increase in female mating frequency, up to a point where male mating capacity becomes limiting [34]; The increased female mating frequency is considered to be a facultative response to the depleted male mating resources in female-biased populations. In *E. mandarina*, on the other hand, female mating frequency did not seem to increase in response to the shift of sex ratio toward females (figure 2*b*), which may be explained by the difference in male mating capacity between the two species.

(d) Costs of *w*Fem infection

Although we could not detect a significant difference in the number of offspring between wild-caught C females and CF females ($p = 0.748$ by GLM; electronic supplementary material, table S1), our data on the wing size suggest that *w*Fem has some physiological cost. Although female wing size tended to be larger in June than in September, which may be explained by the prolonged hormonal events at lower temperature [35], the wing size was significantly smaller in CF females than in C females ($p = 0.042$ by GLMM; electronic supplementary material, table S4). Narita et al. [22] also found that offspring of CF females require a longer development time than offspring of C females. By performing controlled experiments in future work, we may be able to clarify some negative effects of *w*Fem on the fitness-related parameters.

We also expected that the effects of *w*Fem infection on the female body size may act negatively in sexual selection. However, the proportion of nonvirgin females and the numbers of spermatophores were not significantly different ($p = 0.599$ for the proportion of virgins; $p = 0.658$ for the numbers of spermatophores by GLMM), although their pooled values and medians were both higher in CF females than in C females (electronic supplementary material, table S5). Even if CF females were subtly discriminated against by males, such effects on sexual selection would be too small to explain the population dynamics of CF and C females in the population.

(e) Maintenance of *w*Fem in the population despite the absence of suppressors

Because the all-female trait of CF matrilines is not due to male killing, the number of daughters produced by CF females was nearly twice as much as that produced by C females (electronic supplementary material, table S1; [23,24]). Vertical transmission efficiencies of *w*CI and *w*Fem would be high because both *w*CI and *w*Fem were detected by PCR from all of the 40 daughters from each of the three all-female broods produced by females collected in 2007 ($n = 120$) as well as from 12, 14, 11 and 11 daughters from the four all-female broods produced by females collected in 2015 ($n = 48$). Therefore, the fitness cost of *w*Fem infection, as has been suggested above, may act against the increase and fixation of *w*Fem in the population.

Alternatively, underrepresentation of CF females might be explained by the periodical immigration of C females onto Tanegashima Island from other populations. The effects of invading C females may be significant under an extremely small population size resulting from the spread of CF females. CF females would then increase in its frequency, followed by the subsequent decline of population size. Then, the immigration of C females would become effective. If this is the case, the immigration of C females might effectively act against population extinction on Tanegashima Island. It would thus be of interest to examine the genetic diversity of *E. mandarina* in the Tanegashima Island population as well as other populations without CF females to determine the validity of these distinct hypotheses. If the migration is very rare, this population may suffer from substantial inbreeding resulting from the sex ratio bias (i.e., a small effective population size).

(f) Conclusion

Despite our initial expectation of either population decline/extinction or spread of suppressors, the polymorphism (i.e., coexistence of C and CF females) appears to have been maintained in the population for at least the last 12 years (more than 48 generations [36]) without any evidence of suppressors. However, longer-term effects of the female-biased population sex ratio are elusive. If the Tanegashima population is genetically isolated, it could bring some genetic changes in the mating behaviour of males and females [33]. It is also possible that suppressors against female drive may evolve in the future [8,12]. If suppressors emerge, the cost of *w*Fem would lead CF females to decline. Because the spontaneous loss of *w*Fem would lead to all-male offspring [20,24], polymorphism in female karyotypes (WZ and Z0) would not be maintained in the absence of *w*Fem.

If the Tanegashima population is constantly invaded by C females, on the other hand, cyclic fluctuation of the population sex ratio can be explained. In any case, we speculate that the current polymorphism is unstable in the long term, and the population may eventually return to a single infected status (*w*CI-only state) in the future.

Declarations

Authors' contributions

DK, SN and MN designed the study. DK, SN, TK, MM, JA, WM and MN collected data. DK analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Figure 1. Sex ratios of *E. mandarina* on Tanegashima Island. (*a*) Fluctuation of observed sex ratios (i.e., proportions of females among captive individuals; given in red) and deduced sex ratios (i.e., proportions of females deduced from infection frequencies of *w*Fem; given in blue) examined in 13 visits from 2005 to 2017. Four visits where males were overrepresented in the observed sex ratios are indicated by red arrows. Three visits where males were underrepresented in the observed sex ratios are indicated by blue arrows. Asterisks indicate a significant difference between observed and deduced sex ratios by chi-squared test, which compared the actual numbers of captive males and females versus the numbers of *Wolbachia*-diagnosed females (C and CF females) and the virtual number of males (by assuming that it equals to the number of C females) (*$p < 0.05$; **$p < 0.01$ by chi-squared test). (*b*) Correlation between observed sex ratios and deduced sex ratios. Red circles: data of 13 visits to Tanegashima Island. Black rhombi: data of other six populations without *w*Fem. A blue line shows where the observed and deduced sex ratios are equal.

Figure 2. Correlation between sex ratios and mating tendency of *E. mandarina* on Tanegashima Island. (*a*) Proportions of virgin females plotted against observed sex ratios (proportions of females). (*b*) Female mating frequencies (i.e., numbers of spermatophores per female and standard errors) plotted against observed sex ratio. Plots for females collected in June are represented by blue circles and those collected in September by red squares.

Table 1. Number of spermatophores possed by wild-caught *E. mandarina* females

	Proportion of females	No. of females with *n* spermatophore(s)							Proportion of virgin females	Mean no. of spermatophores per female
		n = 0 (virgin)	*n* = 1	*n* = 2	*n* = 3	*n* = 4	*n* = 5	*n* = 6		
(a) Tanegashima population										
Tanegashima (Sep, 2005)	1.000 (42)	25	9	0	0	0	0	0	0.735	0.265
Tanegashima (Jun, 2006)	0.811 (53)	17	21	2	1	0	0	0	0.415	0.683
Tanegashima (Sep, 2006)	0.936 (109)	32	48	18	0	0	0	0	0.327	0.857
Tanegashima (Jun, 2007)	0.676 (34)	3	14	3	0	0	0	0	0.150	1.000
Tanegashima (Sep, 2007)	0.947 (57)	20	25	6	2	0	0	0	0.377	0.811
Tanegashima (Sep, 2009)	0.845 (58)	6	33	7	3	0	0	0	0.122	1.143
Tanegashima (Jun, 2013)	0.865 (74)	35	25	2	0	0	1	0	0.556	0.540
Tanegashima (Jul, 2016)	0.868 (38)	16	16	0	1	0	0	0	0.485	0.576
Tanegashima (Aug, 2017)	1.000 (24)	19	3	0	1	0	0	0	0.826	0.261
									0.415 (median)	0.683 (median)
(b) Other populations										
Zao, Miyagi Pref. (Sep, 2006)	0.400 (20)	0	4	1	0	2	1	0	0.000	2.375
Tochigi, Tochigi Pref. (Sep, 2009)	0.262 (61)	0	1	3	3	2	0	1	0.000	3.000
Shodoshima, Kagawa Pref. (Aug, 2011)	0.237 (135)	0	9	8	6	0	0	0	0.000	1.870
Hachijojima (Sep, 2011)	0.460 (139)	3	29	18	10	2	1	0	0.048	1.714
									0.000 (median)	2.122 (median)

figure 1

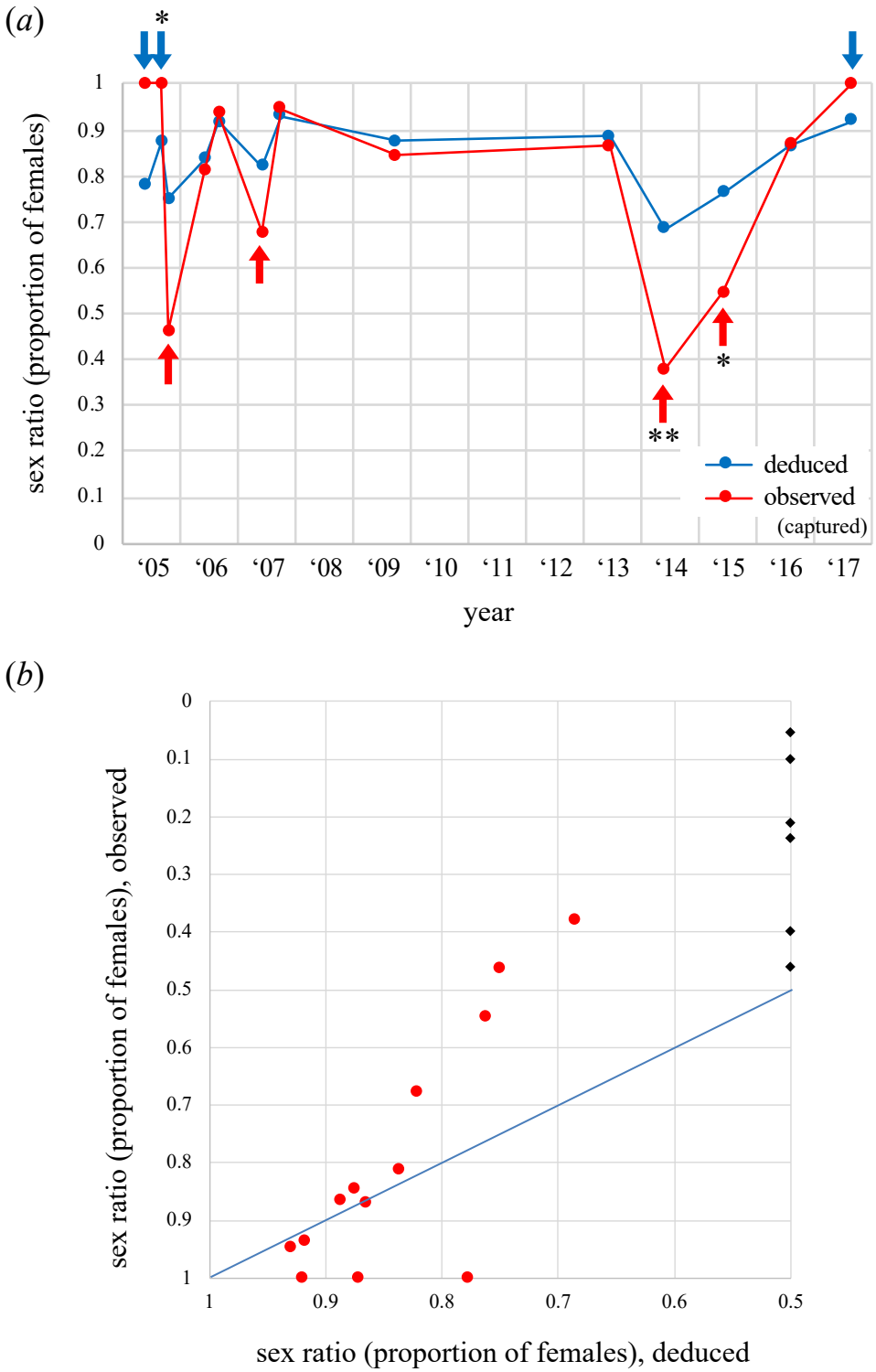
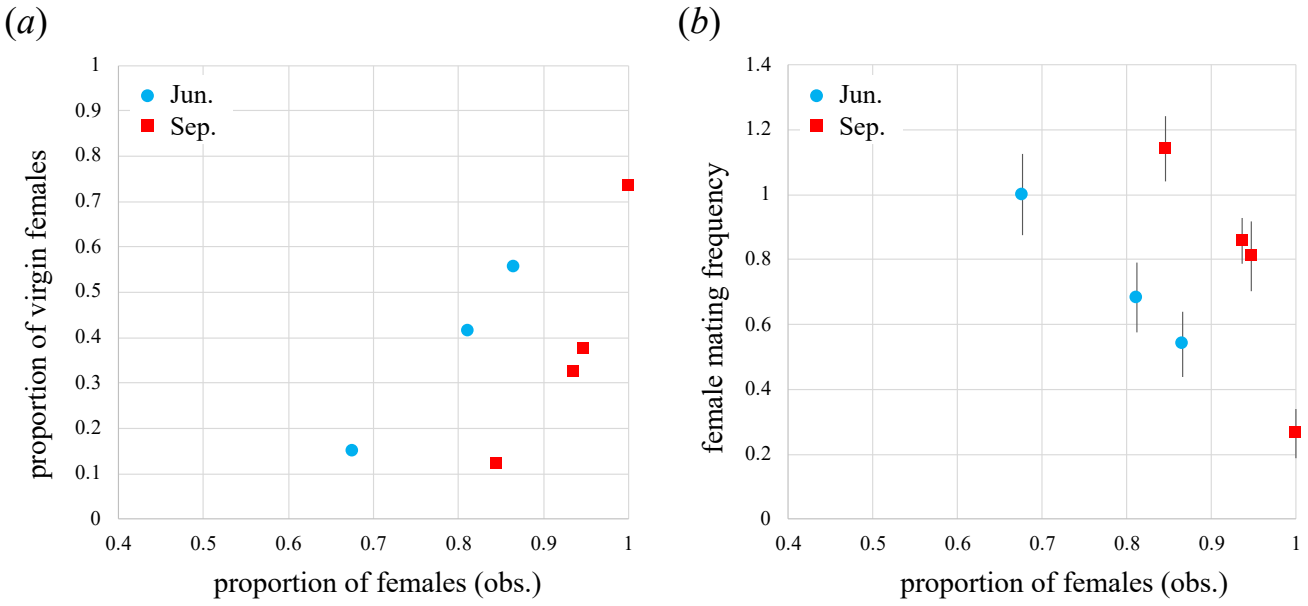

(a)
*
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
sex ratio (proportion of females)
**
*
deduced
observed
(captured)
'05 '06 '07 '08 '09 '10 '11 '12 '13 '14 '15 '16 '17
year
(b)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
sex ratio (proportion of females), observed
1 0.9 0.8 0.7 0.6 0.5
sex ratio (proportion of females), deduced

figure 2


(a)
Jun.
Sep.
proportion of virgin females
proportion of females (obs.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0.4
0.5
0.6
0.7
0.8
0.9
1
(b)
Jun.
Sep.
female mating frequency
proportion of females (obs.)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0.4
0.5
0.6
0.7
0.8
0.9
1