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Sex-ratio adjustment in response to local mate competition is achieved through an alteration of egg size in a haplodiploid spider mite

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Sex-ratio adjustments are commonly observed in haplodiploid species. However, the underlying proximate mechanisms remain elusive. We investigated these mechanisms in *Tetranychus urticae*, a haplodiploid spider mite known to adjust sex ratio in response to the level of local mate competition (LMC). In this species, egg size determines fertilization probability, with larger eggs being more likely to be fertilized, and thus become female. We explored the hypothesis that sex-ratio adjustment is achieved through adjustment of egg size. By using spider mites from a large population, we found that females produced not only a higher proportion of daughters under high levels of LMC, but also larger eggs. Moreover, in populations experimentally evolving under varying levels of LMC, both the proportion of females and the egg size increased with LMC intensity. These results suggest that sex-ratio adjustment in spider mites is mediated by egg size, although the causal relationship remains to be tested.

Keywords: local mate competition; sex-ratio adjustment; egg size; proximate mechanism; haplodiploidy; *Tetranychus urticae*

1. INTRODUCTION

According to sex allocation theory, parents should adjust their relative investment into daughters and sons, depending on which sex has the highest fitness value [1,2]. This prediction has been supported by many empirical studies, and has become one of the greatest successes in evolutionary biology [3]. Adaptive sex-ratio adjustments in response to environmental conditions have been observed in a variety of species, including vertebrates, invertebrates and plants [2]. The best-supported hypothesis has been that of local mate competition (LMC), developed by Hamilton [4], which predicts that population structure determines the optimal offspring sex ratio that females should produce. When mating occurs in small groups representing the offspring of just one or few founding females (hereafter called ‘foundresses’), related males compete with one another for mating partners, so that females are selected to produce a higher proportion of daughters in their progeny. As the number of foundresses colonizing each patch increases, competition between related males decreases, so that a more balanced sex ratio is favoured, approaching the sex-ratio equality predicted for large panmictic populations (i.e. populations with random mating) [1,2,4]. The best empirical support

for LMC theory comes from cases in which females plastically adjust their sex ratio in response to a variable number of foundresses [2]. Although such adaptive sex-ratio adjustments have been observed across many taxa, the most striking sex-ratio patterns are commonly seen in haplodiploid arthropods, such as wasps and mites [1,2].

Despite the success of sex allocation theory, sex-ratio adjustments are not yet fully understood. Most studies have concentrated on testing theoretical predictions (expected sex ratios), whereas the mechanistic aspects of sex-ratio adjustment have been largely neglected. Yet these mechanisms (‘how’ questions) are likely to be crucial for fully understanding the adaptive value of sex-ratio adjustment (‘why’ questions). Moreover, they could contribute to explain the variation across species in the amount and precision of sex-ratio adjustment [2,5]. One of the major unknown variables on sex allocation is the physiological mechanism used to control offspring sex. Sex-ratio adjustment is expected to be stronger and more accurate when this mechanism is straightforward and not too costly [2,6].

In vertebrates, chromosomal sex determination and random meiosis have long been assumed to reduce the scope for sex-ratio manipulation. Hence, skewed sex ratios are usually achieved through the costly sacrifice of eggs or embryos [6] (e.g. in humans [7] and in *Eclectus* parrots [8]). In mammals, studies conducted on embryos raised *in vitro* revealed a sexual dimorphism in the expression of molecules that signal pregnancy to the mother (e.g. in bovines [9]), which might facilitate sex-selective spontaneous

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abortions. In humans, sex differences have also been reported in the placental protein human chorionic gonadotropin (hCG) [10]. Concentration of glucose and other nutrients during early cell division might also influence secondary sex ratio, as higher levels of glucose favour the development and the implantation of male blastocysts, providing a potential mechanism for sex-ratio variation in relation to maternal condition (in bovines [11] and in mice [12,13]). Over the past few years, there has been increasing evidence that females are also able to control the sex of their offspring at conception, thus overcoming the rigid process of chromosomal sex determination (e.g. in birds [14]). The precise mechanisms by which such control is achieved remain unclear, but studies in both mammals and birds (e.g. in macaques [15] and in zebra finches [16]) suggest that it could be mediated by the level of hormones, such as testosterone, circulating in the breeding female around the time of sex determination. In some mammalian species in which males are the heterogametic sex, testosterone concentration in females is assumed to affect the probability of egg fertilization by Y-chromosome-bearing spermatozoa [15]. In birds, in which females are the heterogametic sex, maternal concentration of testosterone is rather hypothesized to act on chromosome segregation during meiosis [16]. Some studies suggest that variations in maternal hormones are likely to be related to the social and environmental variables that have commonly been associated with offspring sex adjustments (e.g. in gulls [17]).

In invertebrates, especially arthropods, sex-ratio adjustments are often thought to be more easily achieved than in vertebrates [2]. Post-conception sex-ratio manipulations exist, for instance in social hymenoptera, in which workers selectively kill male larvae [18]. However, arthropods are primarily known for their extraordinary ability to adjust their primary sex ratio. Many arthropod species have an arrhenotokous haplodiploid sex determination (whereby males develop from unfertilized eggs, whereas females develop from fertilized eggs), which gives females the potential to precisely control the sex of their offspring at conception, according to whether or not they fertilize eggs [1,6]. For example, in honeybees, reproductive males and workers (i.e. females) are reared in differently sized cells, so that it is possible to know which type of egg a queen 'intends' to lay. Eggs collected from small cells are always diploid (i.e. female), whereas eggs collected from large cells are always haploid (i.e. male), suggesting that queens can precisely control whether the eggs they lay are fertilized or not, without any 'error' [19]. However, in most species, how females manage to vary the proportion of fertilized eggs remains puzzling. The mechanism that is most commonly invoked is female control over the quantity of sperm released from their spermatheca. Consistent with that, observations of oviposition behaviour in parasitoid wasps have shown two distinct behavioural sequences in the laying of male and female eggs: the deposition of fertilized (female) eggs is associated with a pause in abdominal contractions, during which sperm is supposed to be released (e.g. [20–22]; but see Lee *et al.* [23], who found that in the sawfly *Athalia rosae*, females exhibited a longer pause when laying an unfertilized egg). However, this kind of study is rare, and in most cases the precise mechanism by which sex ratio is adjusted remains unknown. Hence, although literature abounds with studies supporting sex allocation theory,

the study of the underlying proximate mechanisms is still in its infancy.

Here, we investigate these mechanisms, using the herbivorous haplodiploid spider mite *Tetranychus urticae* as a model species. Previous research with this species suggests that females have a considerable facultative control over their progeny sex ratio, and can adjust it in response to variable LMC levels [24–29]. However, how such skewed sex ratios are achieved remains unclear. In this species, egg size influences fertilization probability, with larger eggs being more likely to be fertilized (and thus become female) than smaller ones [30]. We therefore hypothesize that females use egg size as a mechanism of sex-ratio adjustment. Under this hypothesis, females should produce larger eggs when alone on a patch ('high LMC' conditions), to increase the proportion of daughters among their offspring, whereas they should produce smaller eggs when other females are present on the patch ('low LMC' conditions), to increase the proportion of sons. The general increase in egg size under stronger LMC intensity should lead to larger sizes of both male and female eggs (figure 1a). Alternatively, if, under stronger LMC intensity, females adjust their sex ratio by fertilizing a larger proportion of their eggs without changing the distribution of egg size, the proportion of female eggs should increase under high levels of LMC, but the mean size of both male and female eggs should decrease, whereas the mean egg size should remain constant (figure 1b). Finally, it could be adaptive for females to produce larger offspring when those offspring are likely to be in competition with the offspring of other females (i.e. under weaker LMC intensity). They would then produce larger eggs under 'low LMC' conditions, as egg size correlates with both larval and adult size in this species [30]. Under this last hypothesis, the size of both male and female eggs would decrease with increasing LMC intensity. To compensate for the lower fertilization probability of smaller eggs, females would have to use much more sperm (figure 1c). To test these alternative predictions, we compared both the sex ratio and the size of eggs produced under two LMC intensities ('high' or 'low' LMC) by females from large populations. To further strengthen our conclusions, we then compared the size of eggs sampled in populations that were constrained with different LMC intensities during more than 14 generations, and which have evolved sex ratios that are very close to those predicted by LMC theory [29]. By testing a new mechanism by which females might control the sex of their offspring, this study may contribute to a better understanding of sex-ratio adjustment in haplodiploid species.

2. MATERIAL AND METHODS

(a) Populations of spider mites

(i) Base populations

A large population of spider mites (*T. urticae*, Koch) was established in September 2007 on cucumber plants (variety: Ventura provided by Rijk Zwaan France), under controlled conditions (25°C, photoperiod of 16 L:8 D regime) at the University of Montpellier. This population was established from approximately 5000 individuals sampled from a spider mite population kept on cucumber plants at the University of Amsterdam, and originally collected from a cucumber greenhouse in Pijnacker, The Netherlands, in May 1994.

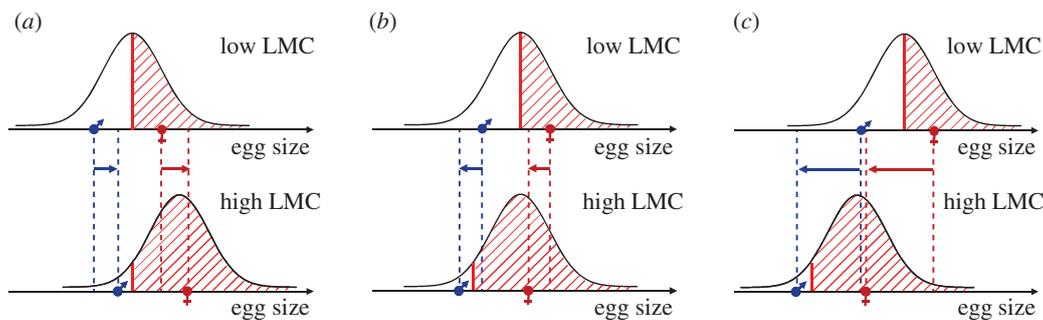


Figure 1. Three possible scenarios for sex ratio and egg size adjustment in spider mites. (a) Female spider mites use egg size to adjust their sex ratio: they produce larger eggs under high LMC than under low LMC intensity, resulting in a higher proportion of fertilized (female) eggs. Here, both male and female eggs should be larger under high levels of LMC. (b) Females adjust their sex ratio by fertilizing a larger proportion of their eggs without changing the distribution of egg size. Here, the proportion of female eggs should increase under high levels of LMC, but the mean size of both male and female eggs should decrease, whereas the mean egg size should remain constant. (c) Females produce larger offspring when those offspring are likely to be in competition with the offspring of other females (i.e. under lower LMC intensity). Here, mean egg size should decrease under high LMC intensity. To compensate for the lower fertilization probability of smaller eggs, females would have to use much more sperm, and the mean egg size of both male and female eggs would both dramatically decrease with increasing LMC intensity. Hatched areas correspond to fertilized eggs; male and female symbols to the mean size of male and female eggs, respectively; blue and red arrows to changes in male and female egg size, respectively, when the intensity of LMC increases.

In September 2008, two independent populations (hereafter called ‘population 1’ and ‘population 2’) were derived from the base population (still on cucumber plants), with approximately 500 individuals each. After a few generations of population growth, each population was composed of several thousands of individuals. In February 2011, two additional populations (hereafter called ‘population 3’ and ‘population 4’) were created from 400 individuals sampled from both populations 1 and 2 and pooled. All four populations were used in this study after about 10 generations of independent evolution.

(ii) Local mate competition populations

To test whether sex allocation evolves in response to LMC, an experimental evolution approach was used (see [29]). In March 2009, populations were created from individuals randomly sampled from the base population, and subjected to three types of selection regime representing different levels of LMC: LMC+ (high LMC), LMC− (moderate LMC) and Panmixia (low LMC). Each type of selection regime was represented by three populations (replicates), which were maintained in a climate chamber at 25°C and evolved during 14 (Panmixia) and 54 (LMC+ and LMC−) generations, respectively.

LMC+, LMC− and Panmixia populations were composed of leaf fragments colonized at each generation by 1, 10 and about 100 females, respectively. The size of leaf fragments was adjusted to the number of foundresses, so that the density was the same for all populations (the leaf fragments measured approximately 4 cm² in LMC+ populations, 40 cm² in LMC− populations and 400 cm² in Panmixia populations; see [29] for further details).

In a previous study [29], we showed that, as predicted by LMC theory, the proportion of males increased with decreasing intensity of LMC under which populations had evolved, with average sex ratios of 0.23 (0.20–0.25), 0.45 (0.43–0.48) and 0.50 (0.48–0.52) for the LMC+, LMC− and Panmixia selection regimes, respectively. These observed sex ratios closely matched theoretical predictions. Furthermore, females from LMC+ populations exhibited a reduced ability to adjust their offspring sex ratio in response

to the number of foundresses present on the patch, compared with females from LMC− and Panmixia populations [29].

(b) Experimental design

All experiments were conducted in a growth chamber at 25°C, under continuous light, on cucumber leaf fragments maintained on wet cotton.

(i) Sex ratio and egg size under high and low local mate competition conditions

For each base population, 24 quiescent virgin females (of similar age) and 24 males were randomly collected and placed together on a 40 cm² leaf fragment. After emergence, females were left with males for 2 days to ensure fertilization. Then, the 24 females were randomly divided into two groups. Females from the first group ($n = 12$) were assigned to the high LMC condition, in which females were placed individually on 4 cm² leaf fragments. Females from the second group ($n = 12$) were assigned to the low LMC condition, where 12 females were placed together on a 48 cm² leaf fragment. The high LMC condition thus consisted of 12 small leaf fragments, each with one female, whereas the low LMC condition consisted of a single large leaf fragment containing 12 females. The density was similar in both conditions, as there was, on average, a leaf area of approximately 4 cm² per female. We chose to control for density to avoid confounding factors other than LMC (such as food availability) affecting egg size and offspring sex ratio, which is in accordance with Hamilton’s model. The larger leaf fragments used in this experiment were small enough to allow females to move from one extremity of the leaf to the other, and thus to detect the presence of congeners.

Before performing any measurement, females were allowed to ‘acclimate’ to these conditions for 2 days. Then, females from both conditions were allowed to lay eggs during 1 day, and those eggs were measured. For that, we randomly collected five eggs per patch in the high LMC condition and 60 eggs in the low LMC condition, which corresponds to the same mean number of eggs per female in each treatment (i.e. five eggs). Those eggs were randomly placed on a glass slide under a binocular

microscope (lens $2\times$, magnification $40\times$), and photographed using AVT SMARTVIEW software. Pictures of eggs were analysed with OPTIMAS v. 6.5 software, which calculates the projected area covered by each egg. From this area (S), we calculated the egg volume (V), assuming a spherical shape, and hence $V = 4/3 \times \pi \times \sqrt{S/\pi}^3$. After being photographed, eggs were placed individually on 1 cm^2 leaf fragments to complete their development and have their sex determined. These data were used to compare egg size between treatments, and to determine the relation between egg size and offspring sex. As offspring sex could only be determined in adult survivors, the number of offspring for which the sex was known was necessarily lower than 60 individuals per treatment.

To properly compare the sex ratio between treatments, we chose to work with a larger number of offspring. Following egg measurement, females from high and low LMC conditions were placed on new leaf fragments (under the same conditions as described above) for a second egg-laying period of 4 days, and then removed. The eggs were allowed to complete their development, and the sex of the resulting offspring was determined at adulthood to obtain the sex ratio. Analyses concerning sex ratio were conducted on these data.

The whole experiment was repeated four times. Hence, as each of the four base populations was tested four times under both high and low LMC conditions, we obtained a total of 16 points of comparison. However, the sex of those eggs that were measured was individually determined for three replicates only.

(ii) *Egg size in populations that evolved under variable levels of local mate competition*

To determine whether females from LMC+, LMC- and Panmixia populations produced eggs of different sizes, 50 freshly laid eggs were randomly sampled in each population and measured as described earlier. This experiment was conducted after 54 generations of evolution for LMC+ and LMC- populations, and 14 generations for Panmixia populations, at the same time as the measurement of sex ratio.

(c) **Statistical analysis**

All analyses were conducted using SAS software [31]. Interaction terms were first introduced in all models and then sequentially removed when their p -value was above 0.1 (for fixed effects), or when their effects had a null estimate (for random effects).

(i) *Relationship between sex and egg size*

We first checked that, as previously found under high LMC conditions, sex was influenced by egg size within each LMC condition, by performing generalized linear models on sex, with egg size as the independent factor, and population and replicate as random factors. For the high LMC condition, female nested within population and replicate was considered as an additional random factor. We specified a binary distribution of the response variable.

(ii) *Sex ratio and egg size under high and low local mate competition conditions*

Each of the four populations was tested four times (four replicates) under both high and low LMC conditions. For the high LMC condition, each replicate was composed of 12 individual females per population, giving 12 sex-ratio values. However, as there were no individual sex-ratio

values for the low LMC condition (one sex ratio per group of 12 females), we pooled data from high LMC condition by replicate and population. In this way, we obtained a total of 16 sex-ratio values for the high LMC condition (four values per population), and 16 for the low LMC condition. We proceeded in the same way for the analysis of egg size; we calculated the mean egg size per replicate (only three replicates), population and condition and carried out the analysis on these values, thus avoiding pseudo-replication. Each of the 32 sex-ratio values was estimated from 194 to 484 offspring ($n = 315$ on average), and each of the 24 mean egg-size values from 60 eggs.

To compare sex ratio and egg size produced under high and low LMC conditions, we used a generalized linear-mixed model, with condition (i.e. high or low LMC) as a fixed factor, and replicate and population as random factors. We specified a binomial distribution of the response variable for sex ratio, and a normal distribution for egg size.

In order to determine whether egg-size variation affected either male eggs, female eggs or both, we then studied the effect of LMC condition on egg size conditional on sex, using those measured eggs for which sex was individually determined. We first calculated the mean egg size per replicate, population and sex. We then carried out a mixed analysis of variance on egg size, with condition and sex as fixed factors, and replicate and population as random factors. A significant interaction between condition and sex would indicate that male and female eggs would be differently affected by LMC.

(iii) *Effect of local mate competition conditions and egg size on egg-to-adult survival*

To determine whether the differences in tertiary (adult) sex ratio observed among LMC conditions could result from sex-specific or egg-size-specific juvenile mortality, we calculated the number of eggs that survived to adulthood for each population, each condition and each replicate. As offspring sex was determined only in 3 replicates, we obtained 24 survival values (three replicates \times four populations \times two conditions). Then, we compared the survival rate between LMC conditions by performing a generalized linear-mixed model on the proportion of eggs that survived until adulthood, with treatment as a fixed factor, and replicate and population as random factors, specifying a binomial distribution. To determine the consequences of egg size for egg survival, we conducted a generalized linear-mixed model on the proportion of surviving eggs, with average egg size as a covariate, and replicate and population as random factors, specifying a normal distribution. This last analysis was conducted both on the total dataset and separately for each treatment.

(iv) *Effect of local mate competition conditions on the total number of offspring*

In most organisms, including spider mites, egg number is negatively correlated with egg size because of physiological trade-offs [32,33]. To determine whether the number of offspring produced by females differed between LMC conditions, we conducted a generalized linear-mixed model on egg number, with treatment as a fixed factor, replicate and population as random factors. We specified a Poisson distribution of the response variable.

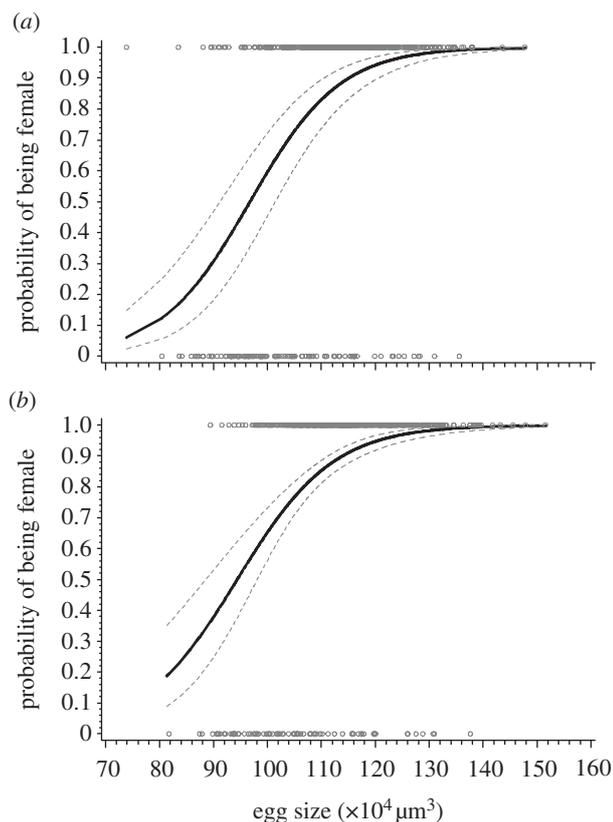


Figure 2. Logistic regression of sex on egg size, under (a) low LMC and (b) high LMC conditions. Under both conditions, larger eggs were significantly more likely to become female than smaller eggs (low LMC: $\log(p/(1-p)) = -11.61 + 0.12 \times \text{egg size}$, $F_{1,569} = 84.09$, $p < 0.0001$; high LMC: $\log(p/(1-p)) = -10.62 + 0.11 \times \text{egg size}$, $F_{1,419} = 53.45$, $p < 0.0001$, with p the probability of being female). Solid lines correspond to mean-predicted values; dotted lines to the 95% confidence limits of the mean-predicted values; individual symbols to observed values.

(v) *Egg size in populations that evolved under variable levels of local mate competition*

To compare the size of eggs produced in the populations evolving under different LMC conditions, we conducted a mixed analysis of variance on the mean egg size per population, with selection regime (i.e. LMC+, LMC− or Panmixia) as a fixed factor, specifying a normal distribution of the response variable. We conducted pairwise comparisons between selection regimes using contrasts.

3. RESULTS

(a) Sex and egg size

Sex was largely explained by egg size, with female offspring more likely to emerge from larger eggs, in both high and low LMC conditions (figure 2). Similar results were obtained when performing the analysis separately for each population ($p < 0.0043$; data not shown).

As predicted by LMC theory, sex ratio was more female-biased under high (18% of sons) than under low LMC conditions (25% of sons; figure 3a). As expected under the hypothesis that females use egg size as a mechanism of sex-ratio adjustment (cf. figure 1), we found that females produced larger eggs under high

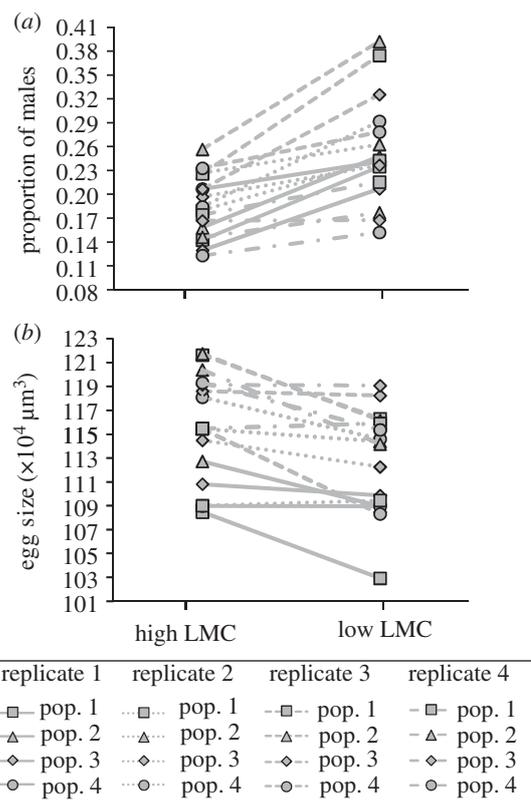


Figure 3. (a) Proportion of male offspring and (b) average size of eggs produced under high and low LMC conditions, in the four populations (four replicates per population). Overall, the proportion of males was significantly higher ($F_{1,3} = 20.26$, $p = 0.021$) and the egg size smaller ($F_{1,3} = 15.08$, $p = 0.03$) under low LMC conditions. Symbols correspond to mean values (the different symbols correspond to different populations, and the different line types to different replicates).

LMC conditions (mean egg size: $115.6 \times 10^4 \mu\text{m}^3$) than under low LMC conditions ($112.8 \times 10^4 \mu\text{m}^3$; figure 3b).

These results show that under high LMC conditions, females produce (i) a higher proportion of daughters and (ii) larger eggs than under low LMC conditions. Egg size was explained by both LMC condition ($F_{1,21} = 6.51$, $p = 0.019$; $F_{1,25} = 6.11$, $p = 0.021$ in the final model) and sex ($F_{1,21} = 100.43$, $p < 0.0001$; $F_{1,25} = 94.39$, $p < 0.0001$ in the final model), but the interaction between sex and LMC condition was not significant ($F_{1,21} = 1.14$, $p = 0.29$), suggesting that the increase of egg size under high LMC conditions impacted both sexes.

(b) Effect of local mate competition conditions and egg size on egg-to-adult survival

The egg-to-adult survival rates were the same in both LMC conditions, with a survival rate of 0.81 (IC: 0.76–0.86) in each condition ($F_{1,17} = 0.02$, $p = 0.90$).

Overall, egg size did not influence egg-to-adult survival, whether the analysis was conducted on the entire dataset ($F_{1,11} = 0.23$, $p = 0.64$) or separately for each LMC condition ($F_{1,5} = 0.03$, $p = 0.87$ and $F_{1,5} = 1.27$, $p = 0.31$, for high and low LMC conditions, respectively).

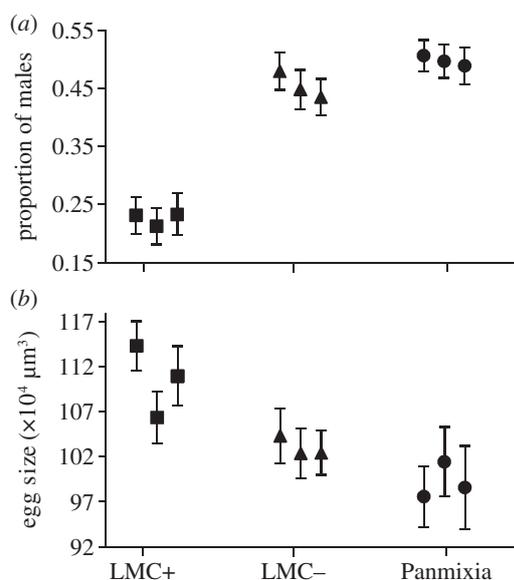


Figure 4. (a) Proportion of male offspring (adapted from [29]) and (b) average size of eggs produced in the LMC+, LMC- and Panmixia populations. The proportion of males was significantly higher in Panmixia populations compared with both LMC- and LMC+ populations ($F_{1,6} = 11.63$, $p = 0.014$ and $F_{1,6} = 355.3$, $p < 0.0001$, respectively), and in LMC- populations compared with LMC+ populations ($F_{1,6} = 242.99$, $p < 0.0001$). Egg size was significantly smaller in Panmixia and LMC- lines compared with LMC+ lines ($F_{1,3} = 27.35$, $p = 0.014$ and $F_{1,3} = 11.96$, $p = 0.04$, respectively), whereas it was similar in Panmixia and LMC- lines ($F_{1,3} = 3.14$, $p = 0.17$). Symbols correspond to mean values (the different symbols correspond to different selection regimes). Error bars correspond to 95% confidence limits.

(c) Effect of local mate competition conditions on the total number of offspring

The number of offspring did not differ between LMC conditions, with, on average, 301 eggs and 320 eggs laid per population and replicate under high and low LMC conditions, respectively ($F_{1,3} = 0.41$, $p = 0.57$).

(d) Egg size in populations evolving under variable levels of local mate competition

The increase in the proportion of males with decreasing intensity of LMC was accompanied by a decrease in egg size, with an average egg size of 110.5, 103.0 and 99.2 for the LMC+, LMC- and Panmixia selection regimes, respectively ($F_{2,3} = 14.15$, $p = 0.03$; see figure 4 for pairwise comparisons).

4. DISCUSSION

(a) Female spider mites adjust their offspring sex ratio in response to local mate competition

Our study confirms that female spider mites are able to plastically adjust their sex ratio in response to the number of foundresses on the patch, in the direction predicted by LMC theory. These results are consistent with previous studies on spider mites [24–29], as well as in other haplodiploid organisms [1,2,5,34,35]. Note that the sex ratios we observed were adult sex ratio, which might differ from primary sex ratios if there is sex-specific juvenile mortality. If this differential mortality is ‘natural’,

for example, if male eggs tend to die more often than female eggs (e.g. because they are smaller), this will only slightly emphasize the difference of sex ratio between high and low LMC conditions (see electronic supplementary material, appendix). However, if mothers sacrifice eggs from a specific sex depending on LMC conditions (e.g. male eggs under high LMC), differences in tertiary sex ratio between high and low LMC conditions could be explained by sex-specific juvenile mortality. However, these hypotheses seem unlikely because survival rates were very similar between high and low LMC.

Although, overall, females produced a higher proportion of sons under low than under high LMC conditions, figure 3a reveals a great variability between populations in the sex ratio produced, and in the slope of the reaction norm. Moreover, the sex ratios observed under low LMC exhibited a large excess of females compared with predicted values (the proportion of males predicted is 0.46 for $n = 12$, calculated from Hamilton’s equation [4], whereas the mean proportion of males observed in our populations under those conditions varied from 0.15 to 0.39, as shown in figure 3, and observed in other studies of spider mites [26,28]). This result contrasts with the perfect match between observed and predicted sex ratios in the populations experimentally evolved under different levels of LMC, which are supposed to be at equilibrium [29]. Possibly, females in populations that are not at equilibrium cannot precisely assess the level of LMC in the patch, or cannot perfectly control their offspring sex ratio at a given time [2]. Another hypothesis that could explain the strong female bias observed under both LMC conditions is that sex ratio was observed at a given time of the females’ life, and not during their whole lifespan. In spider mites, offspring sex ratio varies with females’ age, with a larger proportion of males produced at the very beginning and at the end of life [33]. As a consequence, the sex ratio observed at a given time of a female’s life does not necessarily reflect the sex ratio produced throughout her lifespan.

(b) Egg size as a potential mechanism of sex-ratio adjustment

In this study, we showed that female spider mites adjust not only their sex ratio in response to LMC, but also their egg size. Females produced larger eggs under high LMC conditions than under low LMC conditions, as envisioned by the scenario depicted in figure 1a. Moreover, eggs were found to be larger in populations that evolved under high levels of LMC compared with populations that evolved under lower levels of LMC. To the best of our knowledge, these results constitute the first evidence for an adjustment of a trait other than sex ratio in response to LMC.

A possible adaptive interpretation of these results is that under low levels of LMC, females produced smaller eggs to increase their fecundity, given the often-observed trade-off between offspring size and number [32,36]. However, the total number of offspring did not differ between LMC conditions, suggesting that this hypothesis does not hold. Instead, and according to our initial hypothesis (figure 1a), varying egg size according to the intensity of LMC might be a way for females to adjust their offspring sex ratio.

Indeed, we confirmed the significant relationship between offspring sex and egg size, with larger eggs more likely to be female than smaller ones. Moreover, in a previous study on *T. urticae*, we showed that mothers producing larger eggs when virgin also produced more daughters in their progeny after mating, suggesting that egg size determines fertilization probability, and thus offspring sex [30]. In spider mites, fertilization takes place directly in the ovarian cavity [37–39]. Thus, egg size might influence the probability of fertilization by varying the target size for sperm, so that larger eggs might be more likely to encounter sperm, a phenomenon reported in marine invertebrates with external fertilization [40]. However, the very steep relationship between egg size and sex (figure 2) might indicate that the higher fertilization rate of large eggs does not only result from a passive phenomenon, so that fertilization rate disproportionately increases with egg size. It might be that larger eggs actively attract sperm or stimulate sperm activity better than smaller eggs, for example through the production of attractive chemical substances [40]. Increasing egg size with LMC intensity allows females to increase their probability of producing daughters, and thus adaptively adjust their offspring sex ratio (figure 1*a*). This hypothesis is reinforced by the 16 reaction norms of sex ratio and egg size (figure 3), showing that (i) an adjustment of sex ratio without any adjustment of egg size was observed in a single case (population 3, replicate 3) and (ii) the absence of sex-ratio adjustment (four cases: population 4, replicate 1; population 2, replicate 2; population 1, replicate 4; and population 3, replicate 4) was always associated with an absence of egg-size adjustment. Hence, sex-ratio adjustment and egg-size adjustment appear to be highly correlated ($\chi^2_1 = 11.7, p = 0.0006$). Moreover, consistent with the idea that female spider mites use egg size to control their sex ratio, under LMC, mated females increase their egg size over time, contrary to virgins, who keep it constant [33]. In this species, only the first mating is effective for females, and hence the quantity of sperm stored in the spermatheca probably decreases with age [39]; hence, increasing egg size over time might be a way to increase the probability of those eggs encountering sperm, and thus to maintain a high female:male ratio. Note that (unlike in our former study [30]) we found no effect of egg size on egg-to-adult survival, and no differences in survival rate between LMC conditions, dismissing the hypothesis that biased sex ratios could result from egg-size- or sex-specific juvenile mortality.

Figure 3 shows that the adjustment of egg size varies extensively among populations, suggesting a lack of precision, as for sex ratio. Moreover, in one case (population 3, repetition 3), females adjusted their sex ratio but not their egg size, which might indicate that the sex ratio depends on parameters other than egg size, and that sex-ratio adjustment is achieved through a combination of several mechanistic processes. In spider mites, the sperm cells move through the wall of the spermatheca into the haemocoel, where they move towards the eggs in the ovarium [39,41]. Thus, it is unlikely that female spider mites can control sex ratio through the control of sperm release from the spermatheca, as observed in other haplodiploid organisms [39]. Instead, they could vary the quantity of sperm stored in their spermatheca, with potential consequences on overall sex ratio.

Although our results strongly suggest that egg size is used by females to adjust their sex ratio, we cannot make a definite statement about the causal link between

egg size and sex ratio. Indeed, alternative hypotheses might explain our results. In particular, it might be that egg size was affected by a parameter correlated to the level of LMC, and that the maternal control over offspring sex ratio was achieved only through another, yet unknown mechanism. For instance, under low levels of LMC (i.e. several foundresses per patch), females might have suffered from a greater competition for resources, leading to the production of smaller eggs. This hypothesis seems unlikely because we controlled for density in our experiments, so that all females had access to the same amount of resources. Another hypothesis might be that producing larger eggs under high LMC is advantageous, leading to the simultaneous adjustment of both sex ratio and egg size, without any causal link between these two traits. Egg size can dramatically affect offspring fitness, and, depending on environmental conditions, it might be more advantageous to emerge from either a small or a large egg [36]. For example, in the seed beetle, *Stator limbatus*, the fitness consequences of egg size vary among host plants, and females adaptively adjust their egg size in response to the host encountered [42]. In spider mites, egg size is positively correlated with adult size [30]. In arthropods, larger females can usually lay larger, or more, eggs than smaller ones [32,36]. A larger body size is also often advantageous for males, mainly because of sexual selection [43]. In spider mites, competition between males can be very intense, particularly when males are numerous, because females, for whom only the first mating is effective, are available for reproduction for a very short period [39,44]. To enhance their chance of being the first to mate, males guard quiescent females prior to their final moult, and have to resist attempted interference from other males [39,44]; larger males are more likely to win the competition than smaller ones [44,45]. Hence, a large egg size would be advantageous under both LMC and Panmixia conditions, in terms of overall fitness. The advantage of being large might even be stronger under Panmixia, where competition between males is more intense. Hence, it seems likely that the smaller egg sizes found under Panmixia are a way to adjust sex ratio, rather than an adaptation to those conditions.

Our study provides insights into the proximate mechanisms underlying sex-ratio adjustment in haplodiploids. However, further studies are needed to determine precisely the causal link between egg size and sex ratio in spider mites, for instance through a direct observation of ova and sperm in the ovarian cavity, which might rule out, for example, the hypothesis that the amount of sperm differs among females. Moreover, it would be interesting to test whether the sex allocation patterns observed here are maintained in the absence of density regulation. In natural populations, in contrast with our experimental conditions, lower levels of LMC might be associated with a higher female density. Such a higher density could affect both sex ratio and egg size, for instance by enhancing the ability of females to assess the presence of congeners or by generating food limitation. Furthermore, our results raise new questions concerning sexual conflicts. In haplodiploid species, females have long been assumed to control their offspring sex ratio by simply acting upon fertilization rate. In this context, opportunities for males to manipulate the sex

ratio appeared very limited [46]. Here, we show that the mechanism underlying the control of offspring sex might be more fine-tuned than previously thought, so that males might potentially play a role, for instance by transferring substances that stimulate vitellogenesis, as in some butterflies [47].

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