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Sex allocation in a species with paternal genome elimination: the roles of crowding and female age in the mealybug *Planococcus citri*

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ABSTRACT

Background: In species with paternal genome elimination, both sexes are diploid. However, in males the chromosomes inherited from the father are deactivated during early development and eliminated from the germ line. Sex allocation theory predicts that, all else being equal, females should bias their offspring sex ratio towards the sex that competes least with relatives.

Organism: The mealybug *Planococcus citri*, a cosmopolitan pest on a wide range of agricultural and ornamental plant species.

Hypothesis: In mealybugs, females compete locally for resources. To avoid competition among daughters, females should therefore produce a male-biased sex ratio when alone, but a more equal sex ratio when together with other unrelated females. This will result in a rise of the number of female offspring with density. However, competition associated with population density might have different fitness effects for male and female offspring respectively, because females need more resources and have less opportunity to migrate compared with males, selecting for the opposite pattern of sex allocation.

Methods: Measuring sex ratios in an experiment to manipulate the density a female experiences during two life stages.

Results: Females that experienced high density as adults produced more male-biased sex ratios. In addition, the sex ratio females produced was strongly dependent on their age.

Conclusion: Female mealybugs facultatively adjust their sex ratio, but in the direction opposite to that predicted by local resource competition, suggesting that sex-specific fitness consequences of density determine sex allocation in mealybugs.

Keywords: local resource competition, mealybug, sex determination, sex ratio.

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INTRODUCTION

How individuals allocate resources to their male and female offspring is an important reproductive decision that can have significant fitness implications (Fisher, 1930; Hamilton, 1967; Charnov, 1982; West, 2009). For instance, females have been suggested to facultatively adjust their sex ratio to avoid competition among their offspring (Hamilton, 1967; Charnov *et al.*, 1981) and in response to environmental factors that influence the relative fitness of male and female offspring (Trivers and Willard, 1973). There is an extensive body of empirical data based on tests of sex allocation theory, especially for the haplodiploid Hymenoptera, and the data often fit the theoretical predictions well (West *et al.*, 2000, 2005). However, there are fewer data from other taxa. This is an important omission, as it limits our understanding of sex allocation, especially in terms of the role of different sex determination mechanisms on the evolution of sex allocation. Scale insects (Hemiptera: Coccoidea) vary considerably in terms of their genetic and sex determination systems (Nur, 1980; Ross *et al.*, in press), yet most scale insects have very similar life histories (Gullan and Kosztarab, 1997). Sex allocation in scale insects is poorly understood; in most species, either unbiased or female-biased sex ratios are observed, but very little is known about the factors that influence sex allocation and the extent to which sex allocation is facultative (Brown and Bennett, 1957; Nelson-Rees, 1960).

Among the scale insects, probably the best studied is the mealybug *Planococcus citri* (Coccoidea: Pseudococcidae). Like most mealybugs, *P. citri* has a form of paternal genome elimination (Nur, 1980; Schrader, 1921). Both sexes are diploid but in males one of the parental chromosome sets is deactivated (via heterochromatinization) during early development. The deactivated set is always the set inherited from the father (Brown and Nelson-Rees, 1961). Although the deactivated set divides faithfully in all somatic cell lines, it fails to end up in mature sperm because it is destroyed during meiosis. As a result, males can only pass on the genes they inherited from their mother, making *P. citri* effectively haplodiploid in terms of its transmission genetics (Brown and Nur, 1964). This process is presumably partly under control of the mother, who can 'tag' paternal genomes destined for destruction by genomic imprinting (Khosla *et al.*, 2006), but paternal effects have also been observed (Buglia and Ferraro, 2004).

An important argument that is often used to explain the evolution of haplodiploid genetic systems is that it enables control of the sex ratio by the mother (Bull, 1983). Although control of the sex ratio is well established in many truly haplodiploid species (such as the Hymenoptera), it is less obvious in taxa with paternal genome elimination if and how parents control their offspring sex ratio. This is especially important because paternal genome elimination has been previously considered to be an intermediate system in the evolution of haplodiploidy from diplodiploidy (Bull, 1983). Two previous studies reported evidence of sex ratio adjustment in species with paternal genome elimination. First, Nagelkerke and Sabelis (1998) found sex ratio adjustment in accordance with Hamilton's (1967) theory of local mate competition in two mite species with paternal genome elimination, but failed to repeat the results in other populations of the same species (M.W. Sabelis, personal communication). Second, Varndell and Godfray (1996) tested the effect of population density on sex allocation in *Planococcus citri* mealybugs. In mealybugs, adult males are winged and dispersive, while adult females are sedentary and seldom move once becoming adult (Gullan and Kosztarab, 1997). Females often settle close to where they hatched creating competition between related females for food and space. This competition can become intense since mealybugs, like many phloem-feeding Hemiptera, often form very dense colonies on their host plant. Varndell and Godfray (1996) therefore expected females to produce relatively

more sons under high density, based on the specific assumption that increasing density would increase competition between related females. Competition among relatives for resources, termed 'local resource competition', is expected to favour females that limit competition among offspring of the competing sex by producing more of the sex that avoids competition – in this case, males (Clark, 1978; Charnov *et al.*, 1981). Varndell and Godfray tested this hypothesis by varying both juvenile and adult densities. Although they observed the expected effect of juvenile density, the opposite effect of adult density was found with females producing more female-biased clutches at higher densities. Additionally, the sex ratio was female-biased for all treatments.

However, colony density could affect sex allocation in several ways. First, from the point of view of local mate competition theory (Hamilton, 1967), females could be selected to try and avoid competition between siblings for mates by adjusting the sex ratio towards the sex that competes less for mates (i.e. favouring female production, and reducing local mate competition among sons). Since the extent of local mate competition declines when a female's sons also compete with the sons of other females on a patch, a female ovipositing alone is predicted to produce a more female-biased sex ratio than when she oviposits together with other females. Although local mate competition could feasibly influence sex allocation in *P. citri*, the mating system of *P. citri* (with males being the dispersive sex) does not fit the classic local mate competition scenario. Moreover, the sex ratios observed by Varndell and Godfray in their adult density treatment (assuming density is correlated with an increase in the number of mothers contributing eggs to a resource patch) was opposite to that predicted by local mate competition theory.

Second, following the logic of Varndell and Godfray (1996) outlined above, increasing local population density might increase competition between related females for resources, leading to heightened local resource competition and a decrease in female offspring production [as outlined above and tested by Varndell and Godfray (1996)]. This requires that population density exacerbates the interactions between related female offspring. However, if the number of mealybugs (especially non-dispersing female mealybugs) increases because of oviposition by an increasing number of unrelated mothers, colony density might lead to a reduction of local resource competition, as competition will occur more frequently between non-relatives. This will select for a greater production of daughters with increasing density. This latter scenario is perhaps closer to the 'classic' model of local resource competition.

The effects of resource competition resulting from local population density could also influence sex allocation in a third way. Very dense colonies, while ameliorating local competition among related female offspring, may end up reducing the value of daughters compared with that of sons. In mealybugs, males are winged and able to disperse as adults; they also stop feeding after the second instar and might therefore have a better chance of survival under high resource competition. Female mealybugs could therefore be selected to bias their sex ratio towards more males under high-density conditions. This means that population density should influence sex allocation in opposing directions, depending on the importance of local competition among relatives (local resource competition) versus competition more generally for a female offspring's fitness ('global' competition). A crucial factor underlying this dichotomy will be how density influences patterns of relatedness among interacting female offspring. For instance, an increase in the number of related females in a patch will not reduce local resource competition, whereas an increase in unrelated females will. One aspect of the experiment of Varndell and Godfray (1996) that

makes interpretation difficult is that in some of their treatments all individuals were full-sibs, whereas in other treatments the relatedness among individuals was lower. This means that density and relatedness changed in different ways across their experimental design.

Finally, on a completely different tack, dense colonies could even enhance female offspring fitness via local resource enhancement, with greater numbers of mealybugs facilitating the access and acquisition of resources from the host plant (for instance, by debilitating host defences). This was the suggestion Varndell and Godfray (1996) put forward to best explain their data. Moreover, these patterns of selection on sex ratio could interact (West, 2009).

Given these many alternatives and the difficulty of interpreting Varndell and Godfray's results, here we report a renewed attempt to understand the sex allocation behaviour of *P. citri*. We focus first on understanding how the sex ratio is affected by population density and then on the ability of females to facultatively adjust their sex ratios. We repeat the experiment of Varndell and Godfray (1996), manipulating both juvenile and adult female densities. However, in our experiment all treatments contain a mixture of kin and non-kin (in contrast to Varndell and Godfray, where the juvenile low-density treatment consisted solely of full-sibs), thereby controlling to some extent the effect of high relatedness between ovipositing females. From our experimental design, increasing population density in both juvenile and adult treatments will lead to reduced interactions among kin and a reduction of local resource competition, and so we predict that sex ratios will be more male-biased at low density and more female-biased at high density if classic local resource competition theory explains sex ratio in *P. citri*.

If female mealybugs can assess density as both juveniles and adults, then the density information may either be used equally (thus yielding similar sex ratio changes for females reared at similar juvenile and adult densities), or females may assess or use the information differently. For instance, adult density may be a better predictor of the density a female's offspring will actually experience and thus be the most important cue. This could mean that adult density is used for sex ratio decision making while juvenile density is not, or that there is an interaction between the two such that the effect of juvenile density depends on what adult density is experienced. From work in other organisms on information use and information processing with respect to sex-ratio decisions (e.g. Boomsma *et al.*, 2003; Shuker and West, 2004), we predict that adult density will be a more reliable cue and may therefore be the most important for facultative sex-ratio changes.

Finally, in addition to trying to better control relatedness and density, we also increase the density in both the juvenile and adult high density treatments to better mimic the natural population structure (Nestel *et al.*, 1995). Given that *P. citri* sex ratios have been observed to vary across the female lifespan (Nelson-Rees, 1960; Varndell and Godfray, 1996), we consider both the total sex ratio produced and sex ratio with respect to female age.

METHODS

Study organism

Mealybugs (Pseudococcidae) are the second largest family of scale insects, represented by approximately 2000 species (Hardy *et al.*, 2008). They have strong sexual dimorphism, with adult females being wingless and covered with wax, while adult males are winged and usually

smaller. An important aspect of scale insect biology is their requirement for endosymbionts (typically bacteria), which provide essential amino acids absent from their diet of plant fluids (Buchner, 1965). Here we consider the citrus mealybug *Planococcus citri*, a cosmopolitan pest species previously studied in terms of both its sex allocation and sex determination mechanisms. In *P. citri*, adult males are short-lived (a few days at most), not feeding past the second instar. Females, on the other hand, are usually long-lived (up to several months), forming large colonies and producing gregarious clutches. The first instar nymphs ('crawlers') disperse to new feeding sites when the population is overcrowded (Gullan and Kosztarab, 1997). Adult females become immobile and often settle close to where they hatched, while adult males are winged and disperse. *Planococcus citri* has an obligate endosymbiosis with two bacteria: the primary endosymbiont is the β-proteo-bacteria *Tremblaya princeps* (Tremblay, 1989; von Dohlen *et al.*, 2001), while the secondary endosymbiont is a γ-proteo-bacteria that lives inside the primary endosymbionts (von Dohlen *et al.*, 2001). Reproduction is strictly sexual (Schrader, 1922; James, 1937). Females lay their eggs in an ovisac that consists of fibres that protect the eggs and newly emerged crawlers. Females can produce in excess of 500 eggs during their reproductive lifetime. An advantage of studying sex allocation in mealybugs is that it is possible to determine the primary sex ratio, by examining embryos for the presence of the condensed paternal genome in males. The mealybug culture that was used for the experiment was obtained from Mike Copland at Wye College, London, UK.

Culture conditions

All experimental mealybugs were cultured on sprouting potatoes (cultivar Desiree) in large rectangular plastic boxes (3.2 litres in volume) covered with fine mesh. Cultures were kept at 25°C and 70% relative humidity under a 12-h light/12-h dark regime. Under these conditions, they have a generation time (time from oviposition until sexual maturity) of approximately 30 days. The cultures were maintained by transferring an infected potato sprout to a new box with fresh potatoes each month. For the experiment, individual mealybugs were transferred to smaller boxes and raised on a single potato. Potatoes used for the experiment had a single sprout that was cropped to 15 cm and all potatoes were weighed (in grams) at the start of the experiment to control for the effect of food quantity.

Experiment

The experiment consisted of two juvenile and two adult female density treatments, in a fully factorial design, where the density each female experiences was manipulated at both stages of her life. This allowed us to measure the sex allocation response to density during two different life stages independently. In addition, we measured the effect of density on other life-history factors, most importantly female lifespan and reproductive success.

To generate the two juvenile density treatments, females were randomly selected from the mass culture and transferred to fresh potatoes, where they were allowed to lay eggs for one day. Then, the egg masses were removed and placed either in groups of 3 or in groups of 15 in the treatment boxes (rectangular plastic boxes 1.1 litres in volume) to form the low and high juvenile density treatment respectively. The eggs were allowed to hatch and the crawlers to develop until the third instar, when the sexes of the nymphs become distinguishable (20 days after the juvenile treatments were set up). From every juvenile treatment box, third instar females were randomly selected, removed, and transferred either singly or in groups

of 50 females to new boxes to form the solitary and crowded adult treatments, so that every juvenile treatment box gave rise to two adult treatment boxes. In addition, third instar males were similarly collected and isolated in glass tubes (males do not feed after the second instar). Both sexes were allowed to develop further until 10 days after the adult treatment was set up. By this time both sexes had reached sexual maturity. Males were randomly selected and introduced in groups of three to the female boxes to allow mating. After the males were introduced, females were checked every day for the presence of an ovisac. In the high-density treatment, one female was randomly chosen to be the experimental female; she was marked with a marker pen and only this female was used in the experiment to avoid pseudoreplication. As soon as females started ovipositing, eggs were removed with a small bristle, making sure not to disturb the female (because of the ovisac, egg masses could be attributed to individual females in the high-density treatment). Collected eggs were then fixed and stained to determine the primary sex ratio of each clutch (see below). On the first day of oviposition, the size of the female was measured with a calliper (to the nearest 0.1 mm). Females were checked and eggs collected every day until death.

Egg staining

The egg masses were collected and placed on a glass slide. The protecting fibres around the eggs were removed with a small brush and the eggs were transferred to an Eppendorf tube with fixative (Carnoy's fluid, 4 : 3 : 1, chloroform : ethanol : glacial acetic acid). Eggs were kept in the fixative in a fridge at 4°C for 4 days before they were stained. If eggs had to be stored for longer, they were transferred into 90% ethanol and again stored at 4°C. To determine the sex of each embryo, eggs were transferred to a glass slide, stained with DAPI (Sigma D9564, diluted 1 : 1000 in phosphate-buffered saline) and examined under a fluorescence microscope (200×). The differences between male and female eggs can easily be observed because of the condensed paternal chromosomes that form a brightly stained body in the nuclei of male embryos, which is absent in female embryos (see Fig. 1)

Data analysis

All data analyses were performed using the statistical program R (R Development Core Team, 2008) and for linear mixed models the R package nlme was used (Pinheiro *et al.*, 2007). The relationships between the several life-history traits were explored using generalized linear models with Gaussian error structures. The lifetime sex ratios were analysed using generalized linear models with quasi-binomial error structure to correct for over-dispersion. Due to difficulties with model fitting using generalized linear mixed models, the sex ratio data per day were analysed using a linear mixed-model approach with arcsine-square root transformed sex ratios and female identity fitted as a random effect. Throughout, sex ratios are considered as the proportion of offspring that are male. Age effects on clutch size were analysed with a linear mixed-effect model with female identity again as a random effect. Models were simplified by removing non-significant interaction terms first and then non-significant main effects to generate the minimal adequate model (for procedure, see Crawley, 2007).

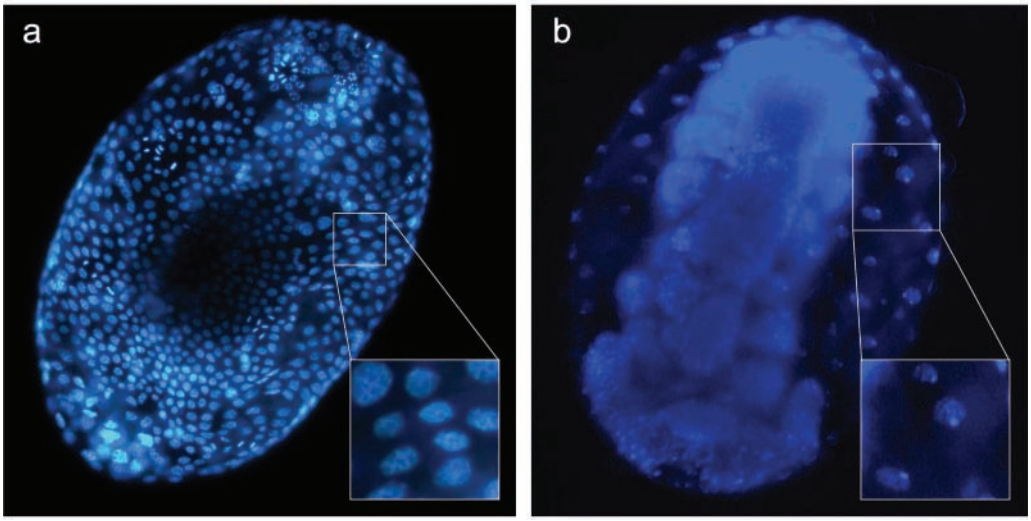

Fig. 1. Mealybug eggs stained with DAPI, examined under a fluorescence microscope at 200× magnification. (a) A female embryo in which the nuclei are uniformly coloured and lack the brightly stained body (see insert). (b) A male embryo in which the condensed paternal genome is visible as a brightly stained body in every nucleus (see insert).

RESULTS

Life history

In total, 48 females commenced ovipositing, producing a total of 22,425 eggs. Of the eggs examined, 97.2% could be sexed successfully. Females lived on average 17.55 days (S.E. = 0.98) following the start of oviposition, and they laid eggs for on average 11.5 days (S.E. = 0.74). During their lifetimes, females laid a mean of 467 eggs (S.E. = 33.96) with a sex ratio of 0.51 (S.E. = 0.014). Female body size was positively associated with lifetime egg production ($F_{1,40} = 50.2$, $P < 0.001$), as was oviposition period ($F_{1,39} = 76.4$, $P < 0.001$). Adult density treatments influenced lifetime egg production, with females producing more eggs when housed at higher density ($F_{1,43} = 5.19$, $P = 0.028$) (Fig. 2). Juvenile density, on the other hand, did not have a significant effect on lifetime egg production ($F_{1,42} = 0.95$, $P = 0.34$); nor did the size of the potato during the two different life stages (potato weight, adult treatment: $F_{1,40} = 0.002$, $P = 0.97$; potato weight, juvenile treatment: $F_{1,41} = 2.62$, $P = 0.11$). There was, however, a significant interaction between juvenile treatment and potato weight during the juvenile treatment on lifetime egg production, with females at high densities producing more eggs when raised on smaller potatoes ($F_{1,38} = 9.04$, $P = 0.005$). Female body size was not affected by either treatment (adult density: $F_{1,42} = 0.01$, $P = 0.90$; juvenile density: $F_{1,43} = 0.01$, $P = 0.93$) or potato weight (potato weight, adult treatment: $F_{1,40} = 0.72$, $P = 0.40$; potato weight, juvenile treatment: $F_{1,41} = 0.79$, $P = 0.38$). There was no significant effect of female size ($F_{1,41} = 1.46$, $P = 0.24$) or potato weight during juvenile development ($F_{1,41} = 0.06$, $P = 0.82$) on the length of the oviposition period, but there was a significant interaction between the two ($F_{1,41} = 4.76$, $P = 0.035$), with a stronger positive effect of potato size in the high-density treatment. There was no effect of the juvenile and

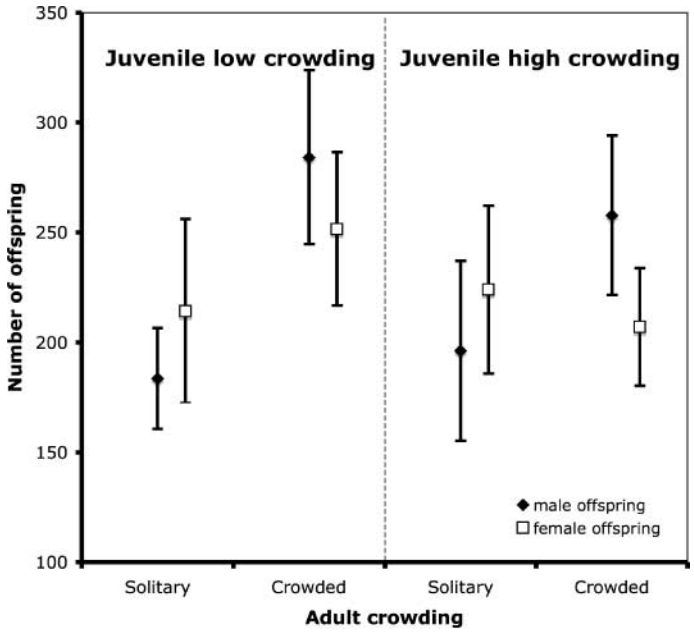

Fig. 2. The average number of male (solid diamonds) and female (open squares) offspring produced per female for each treatment. The error bars are the standard errors. Adult crowding is shown on the x-axis, while the two juvenile treatments are shown in the two vertical panels.

adult density treatments on the oviposition period (adult density: $F_{1,39} = 0.53$, $P = 0.47$; juvenile density: $F_{1,40} = 0.13$, $P = 0.73$).

Sex ratio

The effect of juvenile and adult density treatments on sex ratio was analysed in two different ways. First, we considered the total sex ratio that a female produced during her lifetime using a generalized linear model with a quasi-binomial error structure. There was a significant effect of adult treatment ($F_{1,46} = 8.51$, $P = 0.005$) on the overall sex ratio, with females at high adult densities producing male-biased sex ratios (Fig. 3). There was also a significant effect of potato weight during juvenile development ($F_{1,46} = 15.62$, $P \ll 0.001$) on sex ratio, with females raised on bigger potatoes producing a more female-biased sex ratio. There was, however, no effect of juvenile density ($F_{1,44} = 0.20$, $P = 0.66$), the length of the oviposition period ($F_{1,44} = 3.70$, $P = 0.06$), female body size ($F_{1,44} = 0.55$, $P = 0.46$) or potato weight during adult development ($F_{1,45} = 3.50$, $P = 0.07$). There were also no significant interactions between these factors (all $P > 0.25$).

Second, we considered sex ratios over time. There was a strong effect of the age of a female on the sex ratio she produced (Fig. 4). Females initially produced very male-biased clutches, with their sex ratios becoming more female-biased for approximately 5 days, before the sex ratio started to rise again. Older females produced generally male-biased sex ratios. Therefore, our second analysis considered the effect of treatment on sex ratio per day using a linear mixed-effects model. The results are shown in Table 1 and Fig. 4. There was a strong effect of laying day on the sex ratio of the clutch and the effect was also non-linear (both

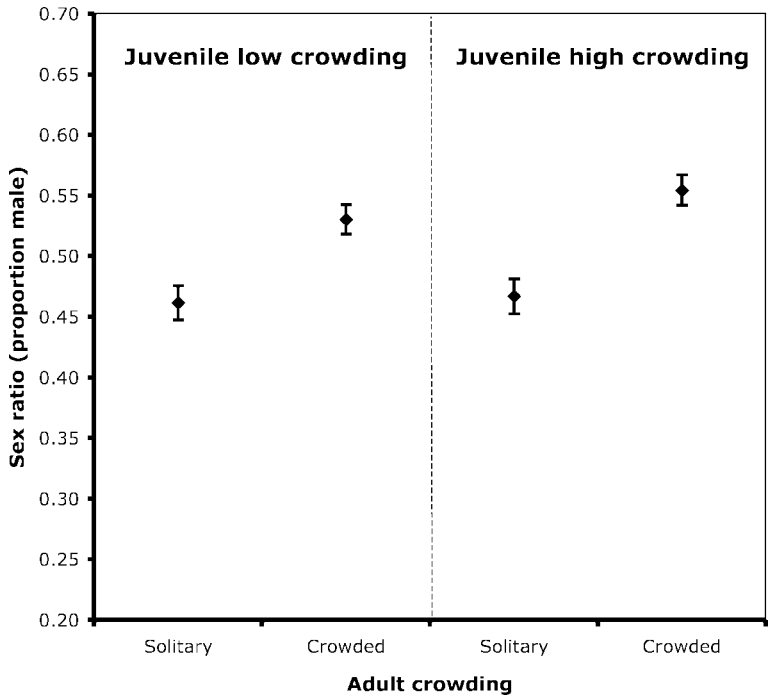

Fig. 3. The average lifetime sex ratio (proportion male) per treatment. The error bars represent the binomial standard errors. Adult crowding is shown on the x-axis, while the two juvenile treatments are shown in the two vertical panels.

Table 1. The minimum adequate model for sex ratio with respect to both treatment and laying day

	Numerator d.f.	Denominator d.f.	*F*-value	*P*-value
(Intercept)	1	483	1237.31	<0.0001
Adult density	1	44	11.41	0.0015
Laying day	1	483	56.38	<0.0001
(Laying day)^2	1	483	135.92	<0.0001
Potato weight JD	1	44	10.40	0.0024
Potato weight AD	1	44	3.44	0.0704
Adult treatment × Day	1	483	4.29	0.0389
Adult treatment × Day^2	1	483	48.71	<0.0001
Day × Potato weight JD	1	483	20.15	<0.0001
Day × Potato weight AD	1	483	12.57	0.0004

Note: The model is a linear mixed-effects model with arcsine-transformed sex ratios and female ID as a random factor. Non-significant interaction and main effects were deleted from the model ($P > 0.05$). JD and AD are juvenile density and adult density respectively.

day and day^2 were significant; see Table 1). Otherwise, the results were largely consistent with the previous analysis. There was also a significant effect of adult density ($F_{1,44} = 11.41$, $P = 0.002$) but not of juvenile density ($F_{1,43} = 0.09$, $P = 0.77$; Fig. 4), and there was a significant effect of potato weight during the juvenile stage ($F_{1,44} = 10.40$, $P = 0.002$) but not

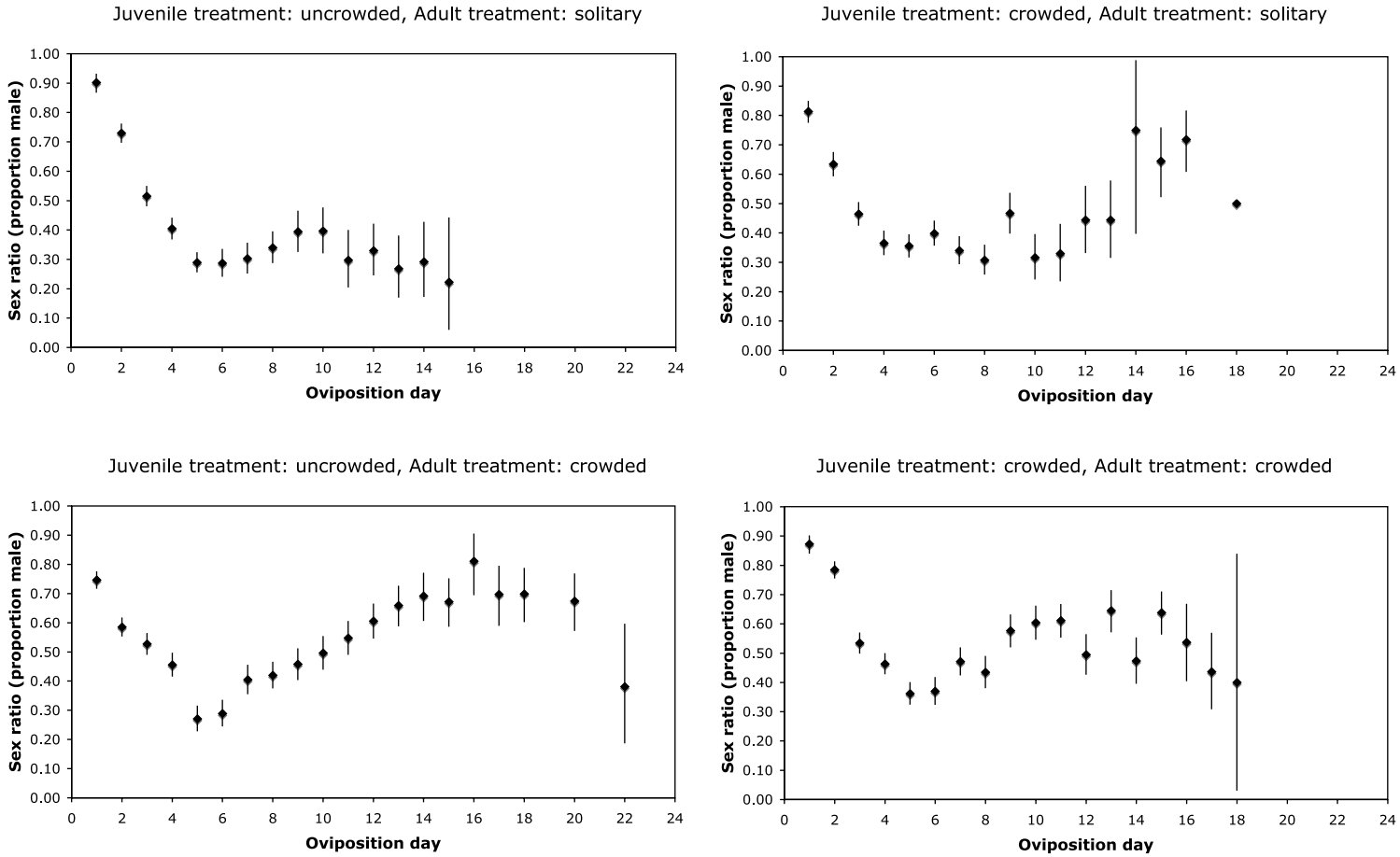

Fig. 4. Sex ratio (proportion male) plotted against oviposition day for each of the four treatment combinations. Error bars show the 95% confidence intervals.

during the adult stage ($F_{1,44} = 3.44$, $P = 0.07$), with females growing on larger potatoes producing a more female-biased sex ratio. There were significant interaction effects between adult treatment and day and day^2 (Table 1), with females under crowded conditions exhibiting a stronger rise in the number of sons they produced late in life (Fig. 4). There were also significant interactions between the potato weight at both life stages and day (see Table 1). Females fed on larger potatoes during the adult stage started to produce male-biased sex ratios earlier and females raised on big potatoes during the juvenile stage showed no rise in number of sons produced, whereas females raised on smaller potatoes did.

DISCUSSION

We found that sex ratio varied with the crowding of the local population, with females producing more male-biased clutches under high adult crowding. This result supports the interpretation that female *P. citri* can facultatively adjust the sex ratio of their offspring and are not constrained by their genetic system. We also found a strong effect of age on the sex ratio produced by females, with females initially producing more male offspring before switching to a female-biased sex ratio, with older females then producing more male-biased sex ratios again (Fig. 4). Despite these dynamic changes in sex ratio, the overall sex ratio was 0.51 (incidentally highlighting the difficulties of interpreting lifetime sex ratios).

The shifts in sex ratios we observed contradict those expected under the classic local resource competition model, which assumes that increasing colony density signifies a reduction in interactions among kin, which it did in our experimental design. When a higher proportion of unrelated females compete for resources (as in our high-density treatments), sex ratios should be less biased, yet we observed a greater male bias under low local resource competition (Fig. 3). However, these results are consistent with density influencing sex ratio through a more general effect on competition among female offspring, such that when densities are high, male offspring will be of greater reproductive value to mothers because they need fewer resources and are able to migrate away from dense colonies.

Varndell and Godfray (1996) tested local resource competition using a slightly different experimental protocol, such that individuals in their high-density adult treatments were either closely related to each other (being derived from one egg sac in the low-density juvenile treatment) or a mix of kin and non-kin (if derived from the five egg sacs of the high-density juvenile treatment). This design risked confounding the effects of competition among relatives (the basis for local resource competition) with (increasingly 'global') competition for resources among unrelated individuals, as increases in density represented by the two adult treatments should have different effects on the extent of local resource competition signalled by high mealybug density. In the treatment where adults were derived from one egg sac, local resource competition remained high and sex ratios should be relatively male-biased (and certainly more male-biased than the high-density treatment drawn from five unrelated egg sacs): the data from Varndell and Godfray (1996) are in the opposite direction. We tried to limit this by generating interactions between less related individuals in the treatments at both the juvenile and adult stages. For us, therefore, increasing density meant a decrease in the degree of competition between related females and so a decrease in local resource competition; in the Varndell and Godfray (1996) experiment, the high adult density treatments included both low and high local resource competition environments. While our current experiment has at least begun to address relatedness and density, we note that further experiments explicitly manipulating relatedness in more detail

may be called for. For instance, high relatedness among ovipositing females could potentially be important for the predicted effect of local resource competition, since their offspring would be related and therefore sex ratios would be predicted to remain male-biased even with increasing population density. Although sex allocation responses to relatedness are not always present [for example, in the parasitoid wasp *Nasonia vitripennis* (Reece *et al.*, 2004; Shuker *et al.*, 2004a, 2004b)], Kasuya (2000) showed that another scale insect, the mango shield scale (*Milviscutulus mangiferae*), does discriminate kin and shows differential migration behaviour to avoid kin competition.

Taken together, however, both experiments question the importance of local resource competition theory for *P. citri* sex ratios, albeit in different ways. In our experiment, increased density led to a reduction in the production of females, suggesting that increased resource competition (but not increased *local* resource competition *sensu stricto*) favours a greater proportion of sons. Perhaps the most likely explanation is that the fitness gains through female offspring decline with increased competition, regardless of interactions with relatives.

We found an average overall sex ratio of 0.51. This again differs from the results of Varndell and Godfray (1996), who found an overall sex ratio of 0.32. Several other studies have looked at sex ratio in *P. citri* and there seems to be substantial variation between the results of these studies (Table 2). Nelson-Rees (1960) found a slightly female biased sex ratio of 0.43, whereas James (1937, 1938) and Schrader (1922) both observed equal sex ratios in their studies. It is of course hard to compare these earlier studies because they tested different things, and were also all based on secondary sex ratios so that effects of differential mortality cannot be excluded. In addition, different authors also used different female densities in their experiments. Table 2 gives an overview of the different studies and the densities used (only adult density is considered because juvenile density is often not stated). However, taken together these results show that the sex ratios observed in the present study are more consistent with the earlier studies, with young solitary females producing an equal or slightly female-biased sex ratio, as compared to the strongly female-biased sex ratios observed by Varndell and Godfray (1996). To better understand the observed differences in sex ratio between different studies and to establish how much of the variation in sex ratios can be explained by genetic differences between populations, we are currently exploring genetic variation for sex allocation among *P. citri* strains drawn from three independent populations.

Why might this general female bias be present? One possibility is that another form of local resource competition, namely local mate competition, is playing a role. Competition among related males is predicted to select for female-biased sex ratios when only one or a few females contribute offspring to a patch (Hamilton, 1967, 1979). However, the observed female bias is small, and given male dispersal in this species, local mate competition among sons is perhaps unlikely to be very important. As mentioned in the Introduction, Varndell and Godfray (1996) offered an alternative explanation for their data. They suggested that perhaps females do not compete with each other, but instead cooperate, possibly because it is easier to obtain resources from their host plant when feeding in groups (i.e. local resource enhancement). However, this hypothesis could not be confirmed in later studies (Varndell, 1995) and it could also not explain the opposite effect of juvenile and adult crowding on the sex ratio. It is also not supported by the data we have presented here.

Local interactions among relatives are not the only potential factors influencing optimal sex allocation in mealybugs, however. Since Varndell and Godfray (1996) published their

Table 2. Sex ratios in studies of sex allocation in *P. citri*: an overview of the available sex ratio data and factors that have previously been shown to influence sex allocation

Author	Sex ratio	Type of sex ratio	Aim of study	Density manipulation	Culture temperature	Age of female when mated
This study	0.46	Primary sex ratio	Effect of density and female age	Solitary as adults; juvenile density varied	25°C	30 days
This study	0.54	Primary sex ratio	Effect of density and female age	Crowded as adults ($n = 50$); juvenile density varied	25°C	30 days
Varndell and Godfray (1996)	0.32–0.38	Primary sex ratio	Effect of density	Solitary as adults; juvenile density varied	25°C	30 days
Varndell and Godfray (1996)	0.25–0.29	Primary sex ratio	Effect of density and female age	Crowded as adults ($n = 20$); juvenile density varied	25°C	30 days
Schrader (1922)	0.47	Secondary sex ratio	Determining sex ratio	Solitary as adult	Unknown	Directly after maturation
James (1937)	0.5	Secondary sex ratio	Effects of female age at mating	Solitary as adult	20.5–26.8°C	Directly after maturation
James (1937)	0.63–0.83	Secondary sex ratio	Effects of female age at mating	Solitary as adult	20.5–26.8°C	Mating delayed 42–70 days after maturation
Nelson-Rees (1960)	0.43	Secondary sex ratio	Effects of temperature and female age at mating	Solitary as adult	20.0–26.0°C	Directly after maturation (41 days)
Nelson-Rees (1960)	0.37–0.84	Secondary sex ratio	Effects of temperature and female age at mating	Solitary as adult	20.0–26.0°C	Varied (25–148 days)
Nelson-Rees (1960)	0.61–0.68	Secondary sex ratio	Effects of temperature and female age at mating	Solitary as adult	29.1–30.2°C	Directly after maturation

Note: Sex ratios were calculated from the raw data presented in the papers or obtained from the figures.

study, the potential for intra- and inter-genomic conflicts over sex ratio has become clear in *P. citri*. For example, recent work by Buglia and Ferraro (2004) suggests that males might be able to control the sex ratio of their offspring as well. This would be selectively advantageous to males because under paternal genome elimination their genes are not transmitted through sons and so males are selected to favour female offspring [similar intra-genomic conflicts over sex ratio exist in true haplodiploids (e.g. Shuker *et al.*, 2006, 2009)]. Moreover, inter-genomic conflict over sex allocation could arise between the host and their endosymbiotic bacteria, since endosymbionts are also only transmitted through females (Normark, 2004). Currently, there is little direct evidence for the influence of either males or endosymbionts on sex allocation (but see below).

In addition to our density treatment effects on sex ratio, we also found that female condition affects sex ratio. First, larger females produced a more female-biased sex ratio, but not more eggs. Second, the size of the potato a female was reared on also influenced sex ratio, with females raised on bigger potatoes producing a more female-biased sex ratio. According to Trivers and Willard (1973), females should adjust the sex ratio according to their condition when one sex benefits more from a mother in a particular condition than the other. The results found in this study suggest that female offspring benefit more from the good condition of their mother. Thus yet another strand of sex allocation theory might also influence mealybug sex allocation. However, Varndell and Godfray (1996) found the opposite pattern, with larger, more fecund and long-lived mothers producing more male offspring. The reason for these differences is not clear.

We also found a strong age effect on the sex ratio that a female produces, with females first producing males, followed by a female-biased sex ratio for several days, and finally producing a more male-biased sex ratio again at the end of their life (although there is an increase in variation of the sex ratios produced by older females). Nelson-Rees (1960) found a similar pattern of early male production for females mated as soon as they reached maturity, although an increase in male production towards the end of a female's oviposition period was only seen when females were prevented from ovipositing for a period. The pattern observed by Varndell and Godfray (1996) falls somewhere in between, with no clear peak of early male production, but a rise in males towards the end of the oviposition period (with some variation among treatments as seen here).

A possible explanation for these observations, and for those from earlier work on temperature (Table 2), is that the sex determination system undergoes changes or deteriorates with age or stress. Kono *et al.* (2008) used quantitative polymerase chain reaction to determine the number of endosymbionts in mealybugs in different developmental stages and found that in older females the number of endosymbionts decreased markedly. Similarly, high temperature leads to a more male-biased sex ratio (Nelson-Rees, 1960), as well as deterioration of the endosymbionts (Buchner, 1965). The extent to which these associations are circumstantial or causal remains to be evaluated, but links to the hypothesis that endosymbiotic bacteria influence sex determination in some way are apparent (Normark, 2004), and we are currently exploring the role of environmental stress on both female condition and sex allocation in more detail.

In summary, sex ratios in *P. citri* are clearly influenced by the environment, both in terms of more direct effects on females (including age and condition) and also in terms of local population density. We have perhaps uncovered more complexity than we have been able to explain, but nonetheless suggest that the role of the environment in altering the value of

female versus male offspring via global competition among females is the most likely explanation for much of the sex ratio data presented here.

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