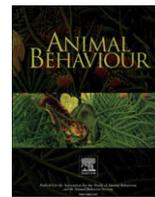




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Extremely female-biased primary sex ratio and precisely constant male production in a parasitoid wasp *Melittobia*

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The study of sex allocation is one of the most productive areas in evolutionary biology, with considerable interplay between theoretical and empirical work. However, observed sex ratios are often measured after developmental periods and they may not reflect primary sex investment ratios, which is what theory predicts. We examined with the sex ratio behaviour of the parasitoid wasp *Melittobia*, in which males do not disperse from their natal patch. In contrast with the well-supported predictions of local mate competition (LMC) theory, the extremely female-biased sex ratio observed at emergence changes little in response to ovipositing female number. We examined (1) the primary sex ratio at oviposition and (2) the pattern of male production over time, to test whether the inconsistency with LMC theory can be explained by differential developmental mortality between the sexes. We found that the sex ratio at oviposition measured with a microsatellite DNA marker did not differ from the sex ratio at emergence, indicating that differential developmental mortality is absent or weak. We also found that males were constantly produced throughout the period of oviposition after a single male was produced initially.

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Sex allocation is one of the most productive areas in evolutionary biology (Charnov 1982; Godfray 1994; Hardy 2002; West, in press). Theory predicts how individuals should divide their investment of resources between male and female offspring in response to environmental conditions, and there are enormous numbers of supporting empirical studies. The most successful area of research into sex allocation has been the study of local mate competition (LMC) in which mating takes place among offspring produced by one or a small number of foundress females, and subsequently only the female offspring disperse (Hamilton 1967). In this situation a female-biased sex ratio is favoured, because it reduces mate competition among related males, and increases mating opportunities (with sisters) for these males (Taylor 1981). In haplodiploids an additional bias is favoured because inbreeding

increases the relatedness of mothers to daughters (Frank 1985; Herre 1985). Consistent with theory, female-biased sex ratios have been reported from a wide range of taxonomic groups in which males disperse less than females (Charnov 1982), and individuals of many species have been shown to adjust their offspring sex ratios facultatively in response to the level of LMC (West et al. 2005).

Most theoretical models predict optimal sex ratios as investment ratios, and so sex ratios should be measured at conception (e.g. at the egg stage in insects). However, most empirical studies on LMC have measured sex ratios at the stage where researchers can visually discriminate the sexes (e.g. adults in insects). These observed sex ratios might differ from the sex investment ratios for many reasons, including differential mortality between the sexes by natural death or differential tolerance for resource competition (Godfray 1986, 1994; King 1993a; Nagelkerke & Hardy 1994), sibling interactions among nestmates (Cockburn et al. 2002), the elimination of sexual caste by workers in social insects (Aron et al. 1994; Sundström et al. 1996) and sex-specific predation or parasitism (Mackauer & Volkl 2005; Pereira & do Prado 2005). Along with the discrepancy between investment and observed sex ratios, developmental mortality is predicted to change the evolutionarily stable (ES) strategy of offspring sex ratio under conditions of LMC. First, developmental mortality in males selects for a less female-biased sex ratio, to reduce the risk of female offspring having no

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males to mate with (Green et al. 1982; Nagelkerke & Hardy 1994; Heimpel 1994; West & Herre 1998). Second, if one sex has a greater negative effect on developmental competition, selection favours a bias in the sex ratio in the direction of the sex having the smaller effect on competition (Godfray 1986; Sykes et al. 2007). Note that these predictions contrast with the panmictic situation, where mortality after the period of parental care has no influence on the ES sex ratio (Fisher 1930; Leigh 1970).

Under conditions of LMC, theory predicts not only overall sex ratios, but also the precision and sequence of the production of male and female offspring (Green et al. 1982; Hardy 1992; Hardy et al. 1998; Krackow et al. 2002). Selection favours mothers that are able to produce offspring sex ratios with a smaller variance than a binomial (random) distribution (called precise sex ratio), because this does not produce superfluous males (Green et al. 1982; West & Herre 1998). The precise sex ratio is achieved by specific sequence orders of sex allocation (Hardy 1992; Godfray 1994; Krackow et al. 2002), and the sequence itself could be an important trait if the emergence order of the two sexes reflects oviposition order (Abe et al. 2005, 2007). For example, asymmetric male competitive ability dependent on emergence order can influence sex allocation (Abe et al. 2003b, 2007; Shuker et al. 2005, 2006).

We examined the sex ratio behaviour of parasitoid wasps in the genus *Melittobia*. Species in this genus have a life history that corresponds to the assumptions of LMC theory, with males that do not disperse, and with mating occurring within their natal host patches before the dispersion of females (Balfour Browne 1922; Hamilton 1967; Dahms 1984; Matthews et al. 2009). In this situation, LMC models predict that foundress females should change their sex ratios (proportion of male offspring) from female biased towards 50% males with increasing number of foundresses (Hamilton 1967; Frank 1985; Herre 1985). However, in contrast to substantial support for this prediction from a range of organisms, especially numerous parasitoid and fig wasps (Herre 1987; Werren 1987; Godfray 1994; Herre et al. 1997; West et al. 2005; West, in press), the extremely female-biased sex ratios of *Melittobia* change little in response to foundress number (Werren 1987; Abe et al. 2003a, 2005; Cooperband et al. 2003; Innocent et al. 2007). One potential explanation for the extremely female-biased sex ratios observed is that there could be enhanced mortality of immature males under high foundress density (Abe et al. 2003a). A high level of lethal combat among adult males is characteristic in this genus and some combatants often die as a result of the combat (Abe et al. 2003a, 2005; Innocent et al. 2007; Reece et al. 2007). However, there is little information about the behaviour of larvae (Dahms 1984; Matthews et al. 2009), especially regarding direct interactions or attacks by adult males. Along with the whole sex ratio, the observed constant emergence pattern of a small number of males over a prolonged period (Abe et al. 2003a, 2005) might be explained by the interaction among developing males of the same cohort.

In this study, our aim was to investigate how mothers allocate their investment to male and female offspring in *Melittobia*. In our first experiment, we measured the offspring sex ratios at oviposition (primary sex ratios) using a microsatellite DNA marker (Ratnieks & Keller 1998; Passera et al. 2001) and compared this with the sex ratios at emergence (secondary sex ratios), to test for differential mortality between the sexes. This allowed us to test: whether (1) the unusual pattern of sex allocation, with little shift in response to the level of LMC, was due to differential mortality; and (2) differential mortality was high enough to influence the ES sex ratios (see above; Nagelkerke & Hardy 1994). In our final two experiments, we investigated the sequence of male and female offspring production by mothers. To exclude the effects of the differences in developmental period among individuals, we removed ovipositing

females from hosts at several time points and created variable brood sizes (Werren 1984; King 1993b; Raja et al. 2008). The sequence of sex allocation could be a potential mechanism to adjust sex ratios with different brood sizes (see Discussion; Herre et al. 1997; Moore et al. 2002; Raja et al. 2008). We specifically examined whether the observed emergence pattern, with small numbers of males constantly emerging over a prolonged period (Abe et al. 2003a, 2005), reflects the oviposition sequence of male and female eggs.

METHODS

Parasitoid Wasps

Melittobia is a gregarious parasitoid wasp of (pre)pupae of solitary wasps and bees nesting above ground (Balfour Browne 1922; Dahms 1984; Matthews et al. 2009). Females have no or just a few eggs at emergence and, after finding hosts, they develop their eggs by feeding on the part of the host on which they lay eggs (synovigeny). Females are potentially able to lay broods of around 500 or more eggs on a single host over several weeks. Eggs laid on a host hatch and start developing from previously laid ones. They are ectoparasitoid, that is, eggs are laid on the surface of the host and hatched larvae develop by sucking the host haemolymph from outside. Male and female eggs show no visible difference in size or in shape (Balfour Browne 1922). There is a pronounced sexual dimorphism in adults. Compared with females, males have small and nonfunctional wings, lack compound eyes and show less pigmentation, and they do not disperse from the host cocoons or host cells. Mating always occurs within their natal patch, at random in respect to matrilineages, when more than one foundress female has laid eggs on a patch (unpublished data). The longevity of adult males depends on mating frequency and they live around 7 days when they mate frequently (Balfour Browne 1922; Dahms 1984). In contrast to males, females have the typical shape of wasps and disperse after mating. There is also dimorphism within adult females: short-wing and long-wing morphs. Whereas the latter females have functional wings, the former disperse on foot. The short-wing females appear in the early part of their emergence bout (Abe et al. 2005), and morph differentiation is thought to be induced by the density of parasitoids and/or the host nutrient quality in their early developmental stage (Freeman & Ittyeipe 1982; Cõnsoli & Vinson 2002; T. M. Innocent, J. Abe, S. A. West & S. E. Reece, unpublished data).

In hymenopteran insects, including *Melittobia*, the haplodiploid mechanism of sex determination allows mothers to control the sex of their offspring by deciding whether the egg is fertilized (females) or not (males; Flanders 1956). However, in contrast to the prediction of LMC models, the extremely female-biased (secondary) sex ratios of *Melittobia* (1–5%) show little change in response to foundress number (Werren 1987; Abe et al. 2003a, 2005; Cooperband et al. 2003; Innocent et al. 2007). There is a large deviation from the prediction: for example, an LMC model predicts 21.4% males when two foundresses lay eggs on a patch (Hamilton 1979). If the foundresses do not experience variable foundress number in the field, the sex ratio shift may not be expected to evolve in this genus (Herre 1987). However, parasitism by more than one foundress is reported from natural populations (Freeman & Ittyeipe 1976; Freeman 1977; van den Assem et al. 1980; Cooperband et al. 2003; Matthews et al. 2009). For example, data from a population of *M. australica* in Jamaica showed that variable numbers of females lay eggs on a host, with an average foundress number of 1.47, producing extremely female-biased offspring sex ratios (Freeman & Ittyeipe 1993).

We used a strain of *M. australica* (Hymenoptera: Eulophidae) that was collected in Shiga, Japan in 2000 (Abe et al. 2005) and prepupae of a leafcutter bee *Megachile sculpturalis* (Hymenoptera: Megachilidae) as hosts. A polymorphism in a microsatellite allele was known for this strain (Abe et al. 2005), and we used it for the identification of sexes of eggs (experiment 1) and for a maternal analysis (experiment 3). Pretreatment to generate foundress females was common to the following three experiments. We collected males and females as pupae from the established microsatellite lines fixed to R or S alleles (experiments 1 and 3) or from a mass culture (experiment 2). We kept female pupae in a group separated from male individuals and isolated male pupae individually to avoid combat. We collected emerged females and males within 24 h after their emergence. We placed eight females and one male into a small plastic case (35 mm in diameter, 20 mm in height) and allowed them to mate for 5 days. We chose this length of time because emerged females ordinarily disperse from their natal patch around 5 days after their emergence (J. Abe, unpublished data). After this mating treatment, we placed the females in a large plastic case (86 mm in diameter, 20 mm in height) with one bee host that was removed from its cocoon, and allowed them to lay eggs. We used long-wing females as foundresses. We conducted all the experiments at 25 °C and a 16:8 h light:dark regime.

Experiment 1

In the first experiment we compared the primary and secondary sex ratios. This experiment used a factorial design with 2×2 combinations incorporating (1) an offspring treatment and (2) a foundress treatment ($N = 6$ for each combination): (1) offspring individuals were either molecularly sexed at the egg stage or morphologically sexed at emergence (sexing timing treatment); and (2) one or two females were allowed to lay eggs on the same host (foundress number treatment). For molecular sexing of eggs using the microsatellite DNA, we allowed a homozygous RR (SS) female to mate with an S (R) male. Heterozygous female eggs (RS) could then be distinguished from males, which had only one maternal allele.

We allowed either one or two females (prepared as above) to lay eggs for 6 days. In the two-foundresses group, the two females had the same genotype. We used RR females in one half of replicates and SS females in the other. After 6 days, eggs laid at the beginning of the oviposition bout had already hatched, and there were eggs and first- and second-instar larvae on the surface of the hosts. At this point, we randomly allocated replicates to one of the following two treatments. Treatment A was conducted to examine the primary sex ratios. We carefully removed all eggs from the host surface under a dissecting microscope and extracted each egg's DNA individually (see below). Because the eggs are very small, it is possible that we might have failed to remove all of them. To check for any oversight of eggs, we also removed all larvae from the host surface and kept the hosts in an incubator. About 15 days later, we checked whether there were developing or emerged individuals. Oversight was very rare with an average of 1.2 individuals (range 0–3) found on a host (all of them female). We did not include these oversight individuals in the analysis. In treatment B, we measured the secondary sex ratios. On the sixth day, we removed all larvae from the hosts, and left eggs on the host surface and allowed them to develop. Once offspring started to emerge, we counted and visually sexed all newly emerged individuals. We removed emerged adults as soon as possible, and if we found killed male adults or late-stage pupae, we also counted them. Because the foundress did not settle or lay eggs on the host in one of the replicates, we removed it from the analysis.

Experiment 2

In the second experiment we determined the sex allocation schedules of male and female offspring production over the whole oviposition period of foundress females. To create different sizes of brood, we experimentally manipulated the duration that females were allowed to lay eggs on hosts. After we introduced one or two females into the large case with a host, we allowed them to lay eggs freely and removed them either 3, 6, 9 or 12 days after their introduction ($N = 8$ for each treatment). We then sexed and removed all newly emerged adults on a daily basis. We also determined the wing morph of females. In three replicates the introduced females did not settle on the host or start to lay eggs normally, and so we removed these from the analysis.

Experiment 3

We conducted the third experiment to examine early oviposition sequences in detail, in particular to reveal whether females produce a male at the beginning of their oviposition bout. The procedure of this experiment was the same as that used for experiment 2, but the oviposition length was shorter and we identified the mothers of all offspring with the microsatellite DNA marker. Once again we had one or two-foundresses in a group. In the two-foundresses group, the two females were from different microsatellite lines. We introduced females into the large case and allowed them to lay eggs on a host for either 48, 60 or 72 h ($N = 12$ for each treatment). To assign the same light and dark periods to all the different oviposition length treatments, we set up all the treatments at the midpoint of the light period. We counted and sexed all emerged offspring. We identified the genotype of all offspring individuals in the two-foundresses group. We discarded any replicates where all introduced females had failed to settle on the host after 48 h or where no offspring emerged.

Molecular Analyses

We applied a boiling method (Kageyama et al. 2006) to extract DNA from eggs. We removed eggs individually from the host surface under a dissecting microscope and put them into 2 μ l drops of 20 mg/ml proteinase K on Parafilm. We squeezed the eggs using the rounded tips of insect pins, mixed them with 50 μ l of a buffer (10 mM Tris, 1 mM MDTA, 25 mM NaCl), and incubated them for 1 h at 55 °C and 10 min at 99 °C. We also extracted DNA from the entire bodies of emerged adult individuals with 100 μ l of the buffer. After centrifuging at 8000 rpm for 1 min, we used the supernatant for polymerase chain reaction (PCR), using the primer set MMS7-2 (Abe et al. 2005). The design of the primers and detailed procedures are described elsewhere (Abe et al. 2005). We identified the PCR products containing (AG)₁₃ or (AG)₁₆ repeat (R or S alleles, respectively) with the silver stain of acrylamide gel following electrophoresis or fragment analysis using ABI GeneMapper (Applied Biosystems Inc., Foster City, CA, U.S.A.) and a capillary sequencer (ABI 3730).

Statistics

We analysed the data with generalized linear models (GLMs) following Crawley (2007) and Wilson & Hardy (2002). We first fitted all the main effects and their interaction terms for each data set and the model was simplified by a backward stepwise procedure. We deleted the terms and tested the significance of changes in deviance until we obtained a minimal adequate model. Brood size data were first analysed with Poisson distributions and the identity link function. However, because the data were

overdispersed (residual deviances > residual degree of freedom), quasi-Poisson errors were used instead. The sex ratio data (proportions of male offspring) and male numbers against brood sizes were underdispersed (residual deviances < residual degree of freedom), indicating precise control of the offspring sex ratios, and so we analysed the data using quasibinomial distributions with the logit link function and quasi-Poisson distributions with the identity link function, respectively. In these analyses using quasidistribution error structures, we assessed significance based on the F statistic (Crawley 2007). In the analysis of male number against brood size, a spurious correlation potentially occurs, because a common term (male number) was contained in both the explanatory and response variables (Jackson & Somers 1991; Brett 2004). To avoid incorrectly rejecting the null hypothesis, we assessed the significance using nonparametric bootstrap methods along with F tests. We determined the P values by randomizations based on 2000 iterations, in which the data of male and female numbers were independently and randomly resampled from the original data. The presence or absence of males in early broods was analysed using a binomial distribution with logit link function, and significances were assessed using chi-square distributions. In this analysis, the interaction terms caused complete separation (the binary responses completely separate at a certain value of a continuous explanatory variable), so we analysed these data including only the main effects. We performed all GLMs with the software package R, version 2.7.1 (The R Foundation for Statistical Computing, Vienna, Austria). We used Kolmogorov–Smirnov tests to compare the emergence pattern of offspring.

Using the data from experiment 2, we tested whether the observed variation of sex ratios is less than a random (binomial) distribution using Green's regression method (Green et al. 1982; West & Herre 1998). This method calculates a ratio (called Green variance) which is obtained by dividing the observed residual variance (mean squared error) of the male number by the expected variance given a binomial distribution. If the ratio is less than one, it means that the observed sex ratios are more precisely controlled than a random distribution. We calculated the mean squared variance and binomially expected variance using a GLM with Poisson distributions and an identity link function rather than normal regression. We tested the significance using the left-hand tail of the chi-square distribution (Green et al. 1982; West & Herre 1998). Our data satisfied an assumption in which the number of males linearly fit the regression to the brood sizes (see below; Krackow et al. 2002).

RESULTS

Primary versus Secondary Sex Ratios (experiment 1)

The examined number of offspring per foundress (analysed egg number in treatment A or emerged offspring number in treatment B) had no significant relationship (Fig. 1a) with sexing timing ($F_{1,21} = 1.44$, $P = 0.24$), foundress number ($F_{1,19} = 0.003$, $P = 0.99$) or genotype ($F_{1,20} = 0.79$, $P = 0.38$). There were also no significant interactions (all $P > 0.085$). We successfully genotyped 1949 (97%) eggs of the 2009 eggs examined in treatment A. The sex ratios were extremely female biased even at oviposition (Fig. 1b). There was no significant effect of sexing timing on the sex ratio ($F_{1,19} = 0.273$, $P = 0.61$), but foundress number showed a significant effect ($F_{1,21} = 7.29$, $P = 0.013$), with females ovipositing alongside other females producing less female-biased sex ratios. However, the sex ratios from the two-foundresses groups were much smaller than the prediction of LMC models. There was a marginal effect of microsatellite genotype (the mean sex ratios \pm SE produced by RR and SS females were 0.018 ± 0.0029 and 0.012 ± 0.0018 ,

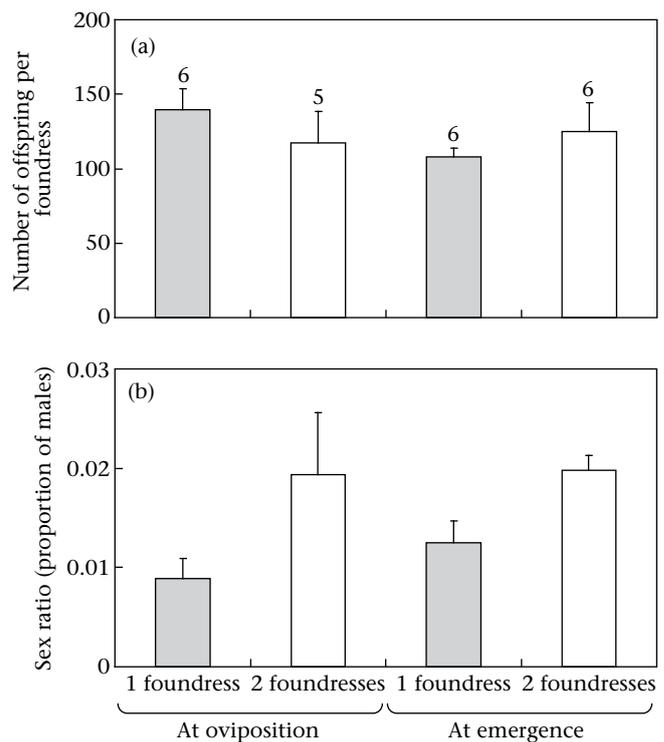


Figure 1. (a) Mean number of offspring per foundress and (b) mean sex ratio (proportion of male offspring) with respect to foundress numbers and timing of sexing. These broods were produced either by one foundress or two foundresses, and examined either before eggs started to hatch (at oviposition) or just after offspring emerged (at emergence). Error bars are SEs and values above the error bars (a) indicate the numbers of replicates analysed.

respectively), although the P value was larger than the 5% significant level ($F_{1,20} = 4.35$, $P = 0.050$). All interaction terms were nonsignificant (all $P > 0.18$).

To examine the effect of the removal of the earliest members on the sex ratio of the later cohort, we also compared the numbers of emerged offspring and the sex ratios at emergence in experiment 1 with the data from the 6-day oviposition length treatment in experiment 2. Although both data sets were obtained under the same conditions, with females allowed to lay eggs for 6 days, we removed the earlier cohorts that had already hatched at the 6th day in experiment 1. The numbers of emerged offspring were significantly smaller in experiment 1 ($F_{1,25} = 43.5$, $R^2 = 0.64$, $P < 0.001$; Figs 1a, 2a), although there were no significant effects of foundress number ($F_{1,24} = 0.13$, $P = 0.71$) or the interactions ($F_{1,23} = 1.38$, $P = 0.25$). The sex ratios at emergence were not significantly different between experiment 1 and 2 ($F_{1,24} = 0.062$, $P = 0.80$; Figs 1b, 2b). The sex ratios from two-foundresses groups were less female biased than those from one-foundress groups ($F_{1,25} = 18.9$, $R^2 = 0.44$, $P < 0.001$), although the interaction was not significant ($F_{1,23} = 0.0001$, $P = 0.99$).

Whole Oviposition Pattern (experiment 2)

Our oviposition length treatments successfully created different brood sizes depending on the oviposition length (Fig. 2a). Brood size per foundress significantly and linearly increased with increasing oviposition length (linear: $F_{1,59} = 479.4$, $R^2 = 0.89$, $P < 0.001$; quadratic: $F_{1,57} = 3.29$, $P = 0.075$; Fig. 2a), but the number of foundresses laying eggs on a host was not significant ($F_{1,58} = 0.53$, $P = 0.47$). There was also no significant interaction between these effects ($F_{1,56} = 0.31$, $P = 0.58$). The cumulative sex ratio decreased

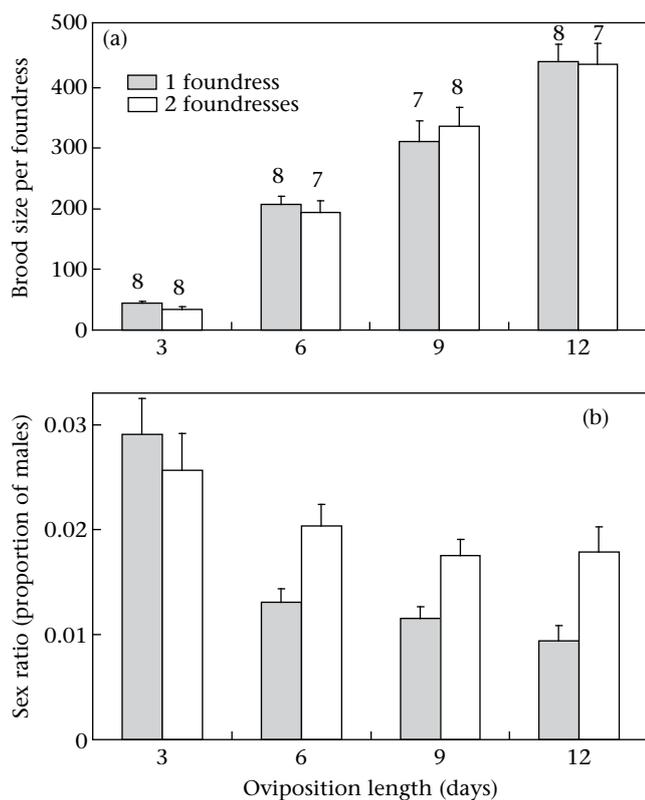


Figure 2. (a) Mean brood size per foundress and (b) mean cumulative sex ratio (proportion of male offspring) with respect to different oviposition lengths for foundresses ovipositing alone or with another foundress. Error bars are SEs and values above the error bars (a) indicate the numbers of replicates analysed.

significantly with increasing oviposition length ($F_{1,59} = 11.6$, $R^2 = 0.11$, $P = 0.001$; Fig. 2b). The sex ratio was higher when females laid eggs with another female than alone ($F_{1,59} = 33.6$, $R^2 = 0.32$, $P < 0.001$; Fig. 2b). There was also a significant interaction between these two factors ($F_{1,57} = 5.35$, $R^2 = 0.05$, $P = 0.024$), such that foundress number had a negligible effect on the sex ratio during the first 3 days, whereas it had larger effects later (Fig. 2b).

We also analysed the number of males against brood size per foundress using the same data set (Fig. 3). In this case, the slope of the regression line represents sex ratio (the proportion of males) at each brood size. There was a significant effect of foundress number ($F_{1,59} = 40.0$, $R^2 = 0.11$, $P < 0.001$) with females from the two-foundresses group producing more males per foundress (Fig. 3). The number of males increased significantly with increasing brood size per foundress ($F_{1,59} = 244.9$, $R^2 = 0.69$, $P < 0.001$) and the increasing rate was significantly steeper in the two-foundresses group (interaction: $F_{1,57} = 42.5$, $R^2 = 0.12$, $P < 0.001$). The quadratic term of brood size was not significant ($F_{1,57} = 0.046$, $P = 0.831$), and plots of the residuals against brood size did not show a specific pattern. This indicates that the relationship between male number and brood size was linear, and the sex ratios were constant independent of brood size. Nonparametric bootstrap methods showed that the effect of spurious correlation was very small and the tendency of the result was the same as the model selection by F statistics (foundress number: $P = 0.009$; brood size: $P < 0.001$; interaction: $P = 0.004$). In addition, when we analysed the regression of male number against female number instead of brood size, the same qualitative result was still found (foundress number: $F_{1,59} = 38.9$, $P < 0.001$; female number: $F_{1,59} = 235.5$, $P < 0.001$; interaction: $F_{1,57} = 42.2$, $P < 0.001$; the quadratic term of female number: $F_{1,57} = 0.11$, $P = 0.744$).

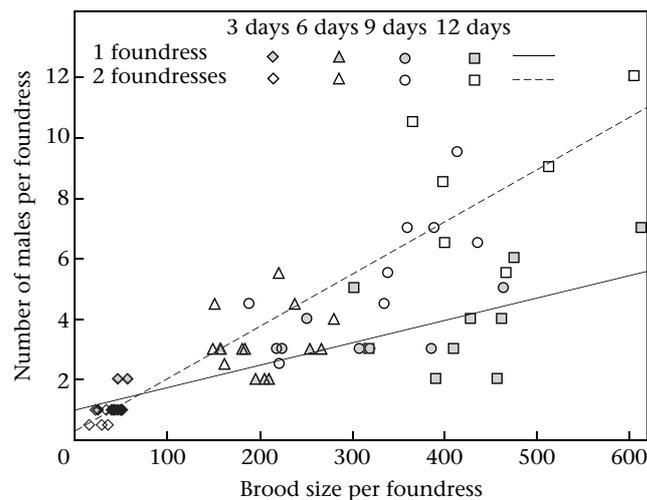


Figure 3. Relationship between brood size and number of male offspring per foundress. The lines represent the minimal adequate model of GLM with Poisson errors and identity link function; one foundress: [male number] = $1.004 + 0.0074 \times$ [brood size]; two foundresses: [male number] = $0.272 + 0.0173 \times$ [brood size].

We tested the precision of sex determination using only the data from the one-foundress group, because we could not recognize individual sex ratios from the data of the two-foundresses group. The Green variance was 0.385 ($=1.08/2.80$), and this was significantly less than one ($\chi^2_{26} = 10.00$, $P < 0.001$). This indicates that the variance of observed sex ratios was less than binomial variation and therefore that the females precisely controlled their sex ratios.

Emergence Pattern and Developmental Period (experiment 2)

We obtained the emergence data of male and female offspring from the treatment in which females were allowed to lay eggs for 12 days. All offspring emerged between 16 and 40 days after the introduction of the foundress females (Fig. 4). The emergence patterns of female offspring were bimodal. The first and second peaks consisted of short-wing and long-wing females, respectively (Fig. 4). Small numbers of male offspring emerged constantly during the whole emergence period except at the end (Fig. 4). Although Fig. 4 represents the mean of male numbers, individual males emerged intermittently in each replicate.

The emergence patterns of each type of morph and for each sex did not differ significantly between the one-foundress and two-foundresses treatments (Kolmogorov–Smirnov test: male: $\chi^2_2 = 0.055$, $P = 0.97$; short-wing female: $\chi^2_2 = 1.34$, $P = 0.51$; long-wing female: $\chi^2_2 = 0.88$, $P = 0.65$). We estimated the developmental periods of males and short-wing and long-wing females by comparing the oviposition and emergence patterns. We used a maximum-likelihood procedure assuming that the developmental periods were normally distributed (see Table 1 for the detailed method). Estimated mean developmental periods of short-wing females were shorter than those of long-wing females (Table 1), although this analysis may contain an artefact because we assumed all short-wing females are laid earlier than long-wing females. Males had a similar developmental period to short-wing females (Table 1). There was little difference in developmental period between the broods produced by one and two foundresses (Table 1).

Early Oviposition Pattern (experiment 3)

Brood size produced by a female increased significantly and linearly with increasing oviposition length (linear: $F_{1,69} = 33.7$,

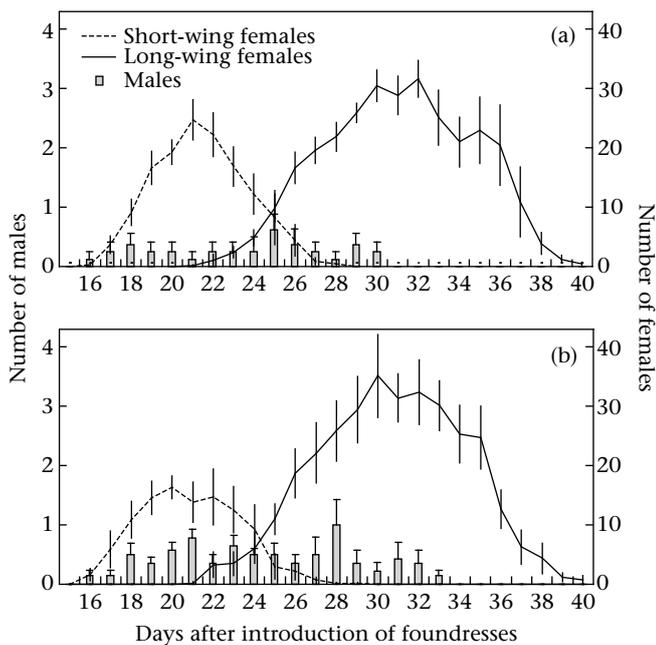


Figure 4. Sequences of the mean number of emerged male offspring and short-wing and long-wing female offspring per foundress, when their mothers were allowed to lay eggs for 12 days. The broods were produced by (a) one foundress or (b) two foundresses. Error bars are SEs.

$R^2 = 0.31, P < 0.001$; quadratic: $F_{1,66} = 0.858, P = 0.36$; Fig. 5). There was no significant effect of foundress number ($F_{1,67} = 0.181, P = 0.67$) or genotype of the microsatellite ($F_{1,68} = 0.622, P = 0.43$). All of the interactions between these effects were also nonsignificant (all $P > 0.21$).

Broods containing no males were small (range 1–14 in one-foundress groups and 2–18 in two-foundresses groups; Fig. 5). In contrast, broods containing one male were larger (range 10–65 in one-foundress groups and 4–41 in two-foundresses groups; Fig. 5). Broods containing two males were rare under the condition of these oviposition lengths. This result indicates that the first male is produced at the beginning of oviposition after some female eggs have been produced, and, subsequently, only females are produced for a while. To analyse the timing of the first male production, the

Table 1
Estimated mean \pm estimated SD of developmental periods (the length between oviposition and emergence) of males and short-wing and long-wing females

	Males	Short-wing females	Long-wing females
One foundress	16.2 \pm 3.6	17.2 \pm 2.2	22.0 \pm 3.3
Two foundresses	16.9 \pm 4.1	17.0 \pm 2.5	22.1 \pm 3.0

We estimated means and SDs by comparing the oviposition pattern obtained from all treatments in experiment 2 with the emergence pattern from the 12-day oviposition length treatment. We used the maximum-likelihood method and assumed the following: (1) males and short-wing and long-wing females have their own developmental periods and the periods are normally distributed; (2) there is no developmental mortality, so that a female produces the same number of eggs as her total emerged offspring number (experiment 1; Fig. 1a); (3) once a female starts oviposition, she lays constant numbers of eggs every day until the end of the oviposition period (experiment 2 and 3; Figs 2a, 5); (4) a female spends the first 2 days finding a host and developing her eggs, and starts oviposition at the end of the second day (the X-intercept [95% confidence intervals] of the regression line of brood size against oviposition length in experiment 2 was 2.01 [1.94, 2.28]; Fig. 2a); (5) sex ratios are constant over the oviposition bout (experiment 2; Fig. 3); and (6) all short-wing females are laid earlier than long-wing females in the same brood (Freeman & Ittjeipe 1982; Cónsoli & Vinson 2002). To obtain the means and SDs of the developmental periods that maximize the likelihood functions, we used the 'optim' function in the software package R.

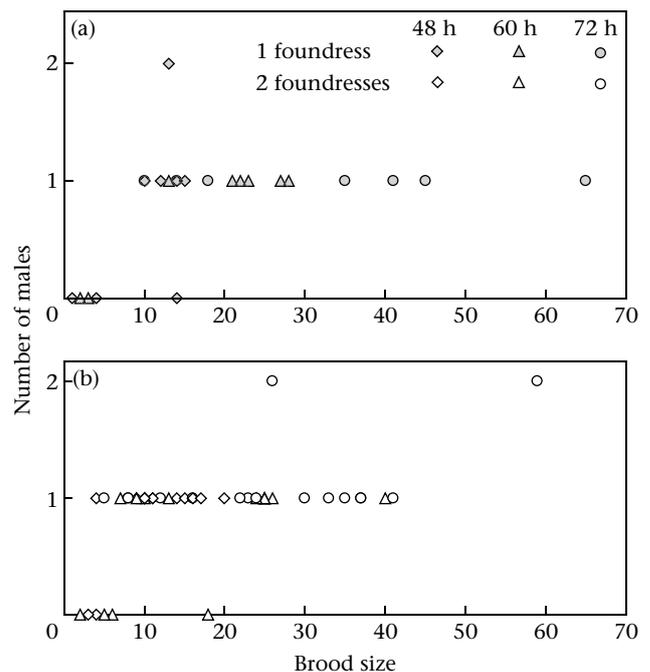


Figure 5. Relationship between brood size and male offspring number per foundress at the beginning of the oviposition period. The broods were produced by (a) one foundress or (b) two foundresses. In the two-foundresses group, the mothers of each offspring were individually identified by a microsatellite DNA marker.

presence or absence of males in the broods was analysed against brood size with a logistic regression. Although the probability of having at least one male increased significantly with brood size ($\chi^2_1 = 27.7, N = 71, R^2 = 0.45, P < 0.001$), the effects of foundress number and genotype of microsatellite were not significant (foundress number: $\chi^2_1 = 1.26, P = 0.26$; genotype: $\chi^2_1 = 0.71, P = 0.40$). The estimated logistic regression line passed through the inflection point at a brood size of 5.6. However, this might be an overestimate of the mean order of the first males, if the data obtained contain mortality of the first males before emergence.

DISCUSSION

We examined the way in which mothers invest their resources into their male and female offspring in the parasitoid wasp *M. australica*. The result of our first experiment suggested that mortality before emergence is very small irrespective of offspring sex and foundress number (Fig. 1). Furthermore, the observed secondary sex ratios are unlikely to be influenced by the members of earlier cohorts (Figs 1b, 2b). Consequently, we can equate the secondary or adult sex ratio with the primary or investment sex ratio.

We also examined the production schedule of male and female offspring. Our second experiment showed that the sex ratio asymptotically decreases with increasing oviposition length and increasing brood size (Fig. 2b). This decreasing sex ratio resulted from the first males being produced early in the oviposition bout (Fig. 5). After the first male, males were produced at a regular interval and their production was precisely controlled over the oviposition bout (Fig. 3). Precise sex ratios and less female-biased sex ratios in shorter oviposition lengths were also reported from another *Melittobia* species, *M. acasta* (Innocent et al. 2007), as well as many other parasitoids (Hardy 1992; Morgan & Cook 1994; Hardy & Cook 1995; Mayhew 1998; Hardy et al. 1998). Many other parasitoid species have been shown to produce males at a regular

position through the period of oviposition, in most cases early or last in the bout (Hardy 1992; Godfray 1994). Whenever males are produced, natural selection favours the production of enough (but the minimum number of) males to inseminate all the sisters emerging from the same patch in the single-foundress case (Green et al. 1982; Nagelkerke & Hardy 1994; Nagelkerke 1996; West & Herre 1998). Furthermore, early male production has advantages, if females cannot predict their brood size before oviposition or if brood production could be disturbed during oviposition (Hardy 1992; Godfray 1994). Our third experiment showed that female *Melittobia* certainly produce one male near the beginning of the oviposition bout, and that this male is not the first or second offspring produced but is produced following several females (Fig. 5). In some parasitoid species with LMC, females lay a female egg first and a male egg second, and this strategy is thought to be adaptive because a single virgin female can still lay male eggs but a single male cannot acquire any reproductive success (Suzuki et al. 1984). In *Melittobia*, virgin females are able to produce both sexes after mating with their own sons (Balfour Browne 1922; Dahms 1984; Matthews et al. 2009) and change their egg-laying behaviour in response to the presence of other mated females (unpublished data).

The sequence of sex allocation can also provide a mechanism for sex ratio adjustment in response to the level of LMC (Herre et al. 1997; Flanagan et al. 1998; Moore et al. 2002; Raja et al. 2008). There are two possible mechanisms for changing offspring sex ratios with foundress number. One is facultative control by mothers in response to foundress number (King 1993b). The other is automatic change by a fixed production sequence decreasing male proportion with increasing brood size, which leads to higher sex ratios when brood production is limited (Waage & Lane 1984; Strand 1988; Herre et al. 1997). Our results showed that the offspring sex ratios decreased with increasing oviposition length, but brood sizes did not differ depending on the foundress numbers. Instead, the sex ratios were facultatively changed over the oviposition bout (Fig. 3). However, observed sex ratios deviated greatly from the prediction of LMC theory when two females laid eggs on the same patch.

Why do females not shift their offspring sex ratios as predicted by LMC theory? First, because emerging males and males just after eclosion are often killed by older adult males and the males have a higher combat ability after this vulnerable period (Abe et al. 2003a, 2005; Innocent et al. 2007; Reece et al. 2007), mothers might be selected to control their offspring sex ratios and offspring production schedules to avoid producing surplus males that would just be killed (Abe et al. 2003a, b, 2005, 2007). A model including this asymmetrical competitive effect predicted that mothers should produce a small number of males over the course of the oviposition period when the competitive asymmetry is large (Abe et al. 2007). Consistent with this prediction, we found that females produce a constant number of males (Figs 3, 4).

In species of the ant genus *Cardiocondyla*, males have ergatoid morphs that fight lethally combat within the natal patch for mating opportunities, and these ants produce the ergatoid males earlier in multiqueen colonies than in single-queen colonies (Yamauchi et al. 2006; Suefuji et al. 2008). This could be interpreted as a race for the production of the first-emerging ergatoid males, because the first males kill all other later-emerging ergatoid males. However, we found no difference in the order of first-emerging males depending on foundress number in the wasp *Melittobia* (Fig. 5). One possible explanation is that *Melittobia* females produce one male early in their oviposition bout even when they lay eggs alone, and so they cannot move the production of this male forward appreciably. Alternatively, females may not be able to detect the presence of other females at the beginning of their oviposition bout (Figs 2a, 3).

In another parasitoid wasp species, females are reported to adjust their offspring sex ratios primarily by detecting the presence of eggs laid by other females rather than the presence of other females (Shuker & West 2004).

Second, if females laying eggs on a patch are related, they may produce more female-biased sex ratios than the LMC prediction. This is because female-biased sex ratios increase the number of potential mates for other related male offspring as well as for their own sons (Grafen 1984; Frank 1985, 1986; Taylor & Crespi 1994; Shuker et al. 2004). Limited dispersal increases the average relatedness between females laying eggs on a host without kin recognition, and this may be likely to be the case in *Melittobia*, because their hosts clump spatially in the field and some females develop as the short-wing morph that disperses on foot. However, limited dispersal also increases the competition for resources among related females, and this effect counterbalances the effect of relatedness when population growth is limited (Bulmer 1986; Frank 1986). Although another type of model, in which more than one generation in a population breeds (haystack model), allows the population to grow without limit until the dispersal generation, the relatedness does not increase with generations, and the difference between the predicted sex ratios and the LMC prediction is negligible (Bulmer & Taylor 1980; Nagelkerke & Sabelis 1996). Recently, some models have shown that additional effects (e.g. overlapping generations and empty sites) destroy the balance between relatedness and resource competition caused by limited dispersal, and allow limited dispersal to favour more cooperative behaviour (analogous to female-biased sex ratios; Taylor & Irwin 2000; West et al. 2002; Grafen 2007; Alizon & Taylor 2008). To test this possible explanation, it would be necessary to construct a specific theoretical model that could be parameterized with data on the natural population structure. Finally, the observed extremely female-biased sex ratios could be explained as 'mutual policing' if females can determine the sex ratio laid by other females and adjust their behaviour accordingly (Kamimura et al. 2008), although no parasitoid is known to be able to do this (Werren 1984; King & D'Souza 2004).

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