The mechanism of sex ratio adjustment in a pollinating fig wasp

Shazia Raja^{1,†}, Nazia Suleman^{1,‡}, Stephen G. Compton^{1,*} and Jamie C. Moore²

¹*Institute of Integrative and Comparative Biology, University of Leeds, Leeds LS2 9JT, UK*

²*Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JT, UK*

Sex ratio strategies in species subject to local mate competition (LMC), and in particular their fit to quantitative theoretical predictions, provide insight into constraints upon adaptation. Pollinating fig wasps are widely used in such studies because their ecology resembles theory assumptions, but the cues used by foundresses to assess potential LMC have not previously been determined. We show that *Liporrhophalum tentacularis* females (foundresses) use their clutch size as a cue. First, we make use of species ecology (foundresses lay multiple clutches, with second clutches smaller than first) to show that increases in sex ratio in multi-foundress figs occur only when foundresses are oviposition site limited, i.e. that there is no direct response to foundress density. Second, we introduce a novel technique to quantify foundress oviposition sequences and show, consistent with the theoretical predictions concerning clutch size-only strategies, that they produce mainly male offspring at the start of bouts, followed by mostly females interspersed by a few males. We then discuss the implications of our findings for our understanding of the limits of the ability of natural selection to produce 'perfect' organisms, and for our understanding of when different cue use patterns evolve.

Keywords: fig wasps; local mate competition; sex ratio; precision of adaptation; cue use

1. INTRODUCTION

Resource allocation to male and female reproduction is predicted to reflect local environmental conditions (Charnov 1982; Hardy 2002). The study of such sex allocation strategies has developed into a highly productive field in evolutionary biology, in part because their close relation to organism fitness also allows quantitative tests of theory (e.g. Orzack 1990; Orzack *et al.* 1991; Flanagan *et al.* 1998; Moore *et al.* 2002, 2005) and so can provide insight into whether there are constraints upon the precision of adaptation (see Herre 1987; West & Herre 1998; Herre *et al.* 2001). An area receiving particular attention has been the evolution of sex ratio strategies (proportion of male offspring produced) in spatially structured mating populations. Hamilton (1967) showed that when females (foundresses) oviposit on their own in these conditions a female-biased sex ratio is favoured because it reduces competition between sibling males for mates (local mate competition or LMC). As the number of foundresses contributing offspring to the mating population increases (and assuming they contribute equally), the potential for LMC decreases and so a less female-biased sex ratio is optimal. This prediction is qualitatively supported by a substantial body of empirical work although the quantitative fit varies between species (see Hardy 2002 and West *et al.* 2005 for recent literature collections). Some authors have posited that, rather than

indicating limits to the ability of natural selection to produce 'perfect' organisms (for discussion of factors likely to constrain adaptation, see Herre 1987; West & Herre 1998; Herre *et al.* 2001), these deviations arise because cues other than foundress density are used to assess potential LMC (West *et al.* 2000, 2002; West & Sheldon 2002; Moore *et al.* 2005). So far, though, experimental support for this argument is limited (but see Shuker & West 2004).

Fig wasps (Hymenoptera, Agaonidae), the pollinators of fig trees (*Ficus* spp., Moraceae), have often been used to investigate the effects of LMC on sex ratio strategies. Their mating ecology corresponds with many of the assumptions of Hamilton's (1967) model. *Ficus* is characterized by its inflorescence, the fig, which is lined on its inner surface with a number of flowers. Fig wasp foundresses enter figs through the bract-lined ostiole, and gall and deposit single eggs into some of the female flowers while pollinating others. The larvae then develop and mature into adults in the galls, and eclose and mate in the fig before the females disperse in search of other figs in which to oviposit. Generally, only a small number of foundresses oviposit in each fig, so the probability of sibling mating is high. Also, fig wasp whole fig (brood) sex ratios are qualitatively consistent with the predictions of LMC theory: in many species, they increase with foundress density (see Herre *et al.* 1997 for a review). In several species, though, quantitative tests have indicated that brood sex ratios are more female biased than predicted by extensions to LMC theory (Frank 1985; Herre 1985; Werren 1987) that incorporate the differences in relatedness to male and female offspring that exist in inbred haplodiploids (such as fig wasps) and assume that

* Author for correspondence (s.g.a.compton@leeds.ac.uk).

† Present address: The Institute of Plant and Environmental Protection (IPEP), National Agriculture Research Centre (NARC), Park Road, Islamabad 44000, Pakistan.

‡ Present address: Entomology Division, Nuclear Institute of Agriculture (NIA), Tando Jam 70600, Sindh, Pakistan.

density is used as a cue (reviewed in Herre *et al.* 1997, 2001; see also Kinoshita *et al.* 1998; Moore *et al.* 2002). In some cases, this is due to data from cryptic species being combined and hence foundress densities being over-estimated (Molbo *et al.* 2003, 2004), but in others data are from single species (e.g. Moore *et al.* 2002). Previously, we have investigated individual foundress (clutch) sex ratios in one of these latter species, *Liporrhopalum tentacularis* Grandi, and have shown that they are (negative logarithmically) related to the size of the clutch deposited, not foundress density in the fig (Moore *et al.* 2005). Foundress strategies closely approximate the quantitative predictions (when appropriately parameterized) of a theoretical model we developed predicting optimal sex ratios when strategies are adjusted according to the clutch size. With this hypothesis, the increases in brood sex ratio with foundress density observed in the species (Moore *et al.* 2002) are posited to occur because foundresses are increasingly oviposition site limited in multi-foundress fig (a consequence of female flower number in figs), and so lay smaller male-biased clutches.

Here, we experimentally test the hypothesis that *L. tentacularis* foundresses adjust their sex ratio according to their clutch size. First, we make use of species ecology to test whether increases in brood sex ratio with foundress density arise solely due to oviposition site-limited foundresses depositing smaller less female-biased clutches in multi-foundress figs, and are not due to any response to foundress density. *L. tentacularis* foundresses (along with many other pollinating fig wasp species) routinely re-emerge from figs after depositing a clutch and go on to enter and oviposit in other figs (Moore *et al.* 2003). We show that foundresses entering their second fig (these are hereafter termed 'wingless' foundresses, since they have almost always lost their wings during entry to the first fig) deposit smaller clutches than those entering their first fig (hereafter termed 'winged' foundresses), and therefore are less oviposition site limited when depositing clutches in figs with other similar foundresses. Given this, we predict that if the brood sex ratio increases with density observed in multi (winged)-foundress figs are solely a consequence of smaller clutch sizes, a similar increase will not occur in figs oviposited in by multiple, less oviposition site-limited, wingless foundresses.

Second, we introduce a new technique to test whether clutch size actually is used as a cue by foundresses determining their sex ratio. Moore *et al.*'s (2005) model predicts that such foundresses should exhibit a negative logarithmic relationship between clutch size and sex ratio. Hence, to maintain the optimal sex ratio as clutch size increases, they should produce mostly male offspring at the start of oviposition bouts, followed by mostly females with a few males interspersed (see also Stubblefield & Seger 1990). However, clutch and brood sex ratios similar to those observed could also be as a consequence of another strategy, that of depositing a number of males at the beginning of oviposition bouts followed by only females (see also Herre *et al.* 1997; Kinoshita *et al.* 2002; Moore *et al.* 2002; Kjellberg *et al.* 2005). To determine whether foundresses use clutch size as a cue or pursue a 'males first' strategy, we quantify the sequence in which they deposit male and female offspring by using insecticide to terminate oviposition bouts at varying times after entry to the fig. We then go on to discuss the implications of our

findings for understanding the constraints upon the precision of adaptation and for the understanding of how and why there is variation in the cues used by different species when assessing potential LMC.

2. MATERIAL AND METHODS

(a) Study species

We used the University of Leeds glasshouse population of *L. tentacularis* and its host *Ficus montana* Blum.f for the experiments. Organisms originate from the Centre for International Forestry Research (CIFOR) plantation, Bogor, West Java, Indonesia (06°34' S, 106°45' E) and from Rakata, Krakatau Islands, Indonesia (06°00' S, 105°27' E) and have been in culture since 1996 (Moore 2001). Approximately six wasp generations are produced each year, totalling more than 60 since population establishment.

(b) Brood size and composition of winged and wingless foundresses

We located figs on trees in the population before they were receptive to oviposition and bagged them to prevent foundress entries. When these figs were receptive to oviposition, we located other figs just before wasp release and placed them in mesh-lidded pots. Following wasp emergence the next day, we allowed one, two or three winged foundresses (i.e. those waiting to deposit their first clutch) from the pots to enter each bagged fig. The figs were then bagged again to prevent further pollinator entries or parasitism by non-pollinating fig wasps present in the glasshouse (see Moore 2001 for details) and left to develop. This experiment was also repeated with wingless foundresses depositing their second clutch. Single foundresses were allowed to enter figs and any that re-emerged over the next few hours were collected and allowed to enter another fig. Again, one, two or three foundresses were allowed to enter each fig. Just prior to the experimental figs maturing, we placed them individually into mesh-topped plastic containers to collect the emerging wasps. Once the wasps had emerged, we counted and sexed them. Our sample sizes for winged foundresses (including data first presented in Moore *et al.* 2002) were 60 single-foundress, 38 two-foundress and 33 three-foundress broods, and for wingless foundresses, 9 single-foundress, 9 two-foundress and 9 three-foundress broods.

(c) Clutch size and composition with differing oviposition bout length

To determine the sex of offspring deposited during specific periods of an oviposition sequence, we killed foundresses at different intervals of time after they had entered the figs. Preliminary tests with the insecticide pyrethrum (pre-diluted in water, Fisons Ltd, U.K.) using 20 foundresses showed that they were consistently killed by contact with 1 µl in less than 1 min. This was repeated under more natural conditions by introducing single foundresses into 20 figs and then injecting the pyrethrum using a very fine syringe through the ostiole of the figs. When the figs were opened all the foundresses were dead. In the third preliminary trial, we examined whether the pyrethrum also affected offspring deposited by foundresses or the figs. We introduced single foundresses and then injected 1 µl of pyrethrum. The figs were then placed in fine cotton bags so that no other wasps could oviposit and left to develop. Out of 10 figs, seven matured normally and contained

Table 1. Brood size and composition in figs entered by different numbers of winged or wingless *L. tentacularis* foundresses (see §2 for sample sizes).

		winged foundresses		wingless foundresses	
	foundress density	mean $\pm$ s.e.	range	mean $\pm$ s.e.	range
brood size	1	62.08 $\pm$ 3.08	16–11	30.22 $\pm$ 7.11	14–83
	2	48.05 $\pm$ 3.09	16–88	39.67 $\pm$ 7.46	9–92
	3	49.39 $\pm$ 3.10	25–106	55.20 $\pm$ 5.66	27–81
brood sex ratio	1	0.09 $\pm$ 0.01	0.01–0.30	0.25 $\pm$ 0.03	0.14–0.43
	2	0.18 $\pm$ 0.01	0.04–0.50	0.25 $\pm$ 0.06	0.09–0.67
	3	0.23 $\pm$ 0.02	0.10–0.41	0.26 $\pm$ 0.03	0.10–0.40
males produced	1	4.83 $\pm$ 0.26	1–9	6.77 $\pm$ 1.01	2–12
	2	8.31 $\pm$ 0.82	3–25	8.11 $\pm$ 1.41	4–15
	3	10.74 $\pm$ 0.82	4–20	13.80 $\pm$ 1.95	6–24
females produced	1	57.25 $\pm$ 2.99	12–110	23.44 $\pm$ 6.32	8–71
	2	39.73 $\pm$ 2.86	10–84	31.55 $\pm$ 6.57	3–77
	3	38.64 $\pm$ 2.83	15–95	41.40 $\pm$ 4.76	17–64

numbers of wasps similar to control figs where no insecticide was introduced. The abortion rates were also typical of bagged figs.

We used this technique to quantify offspring sex ratios and oviposition rates of winged and wingless foundresses during various periods after their entry into figs. Winged and wingless females were obtained as before. Single foundresses were introduced and allowed to oviposit for either 5, 15, 30, 60, 120 or 240 min before we interrupted oviposition by gently introducing a syringe through the ostiole and injecting 1 μ l of pyrethrum solution. The figs were then bagged and processed as before. Our sample sizes for these experiments were for winged foundresses, 5 min 9 broods, 15 min 10 broods, 30 min 10 broods, 60 min 14 broods, 120 min 10 broods, 240 min 17 broods, and for wingless foundresses, 5 min 9 broods, 15 min 10 broods, 30 min 10 broods, 60 min 10 broods, 120 min 9 broods, 240 min 10 broods.

(d) Data analysis

We used a combination of linear models (LMs) and generalized linear models (GLMs) to analyse the data on brood size and composition. The brood size data were normally distributed, so we fitted an LM with foundress type (winged or wingless) and foundress density as fixed factors and also their interaction term. We then found the minimum adequate model (MAM), containing only significant terms, by using *F*-tests to test term significance upon removal from the model (we also tested the significance of differences between foundress densities here, by collapsing factor levels and using *F*-tests to compare models). The test statistics given in the text represent term significance after removal from the MAM. The brood sex ratio data were proportions, so we used a GLM with binomial errors, designating male number in the fig as the response variable and brood size as the binomial denominator. We fitted the same explanatory variables in this model as in the brood size analysis. The male and female number data were non-normally distributed count data, so we used GLMs with Poisson errors, again fitting the same explanatory variables as in the brood size analysis. We found the GLM MAMs as in the LM analysis, this time using likelihood ratio tests to test term significance unless the data were overdispersed, in which case we used *F*-tests.

We used similar techniques to analyse the data on offspring production rates over time. The foundress clutch size and male and female offspring production data were non-normally distributed count data, so we analysed them using GLMs with Poisson error structures, and, as with the brood sex ratio data, specified a binomial error structure with male number as the response variable and clutch size as the binomial denominator when analysing the clutch sex ratio data. In these analyses, we fitted foundress type as a fixed factor, oviposition bout length (log transformed after visual inspection of the data in the clutch size and male and female production analyses) as a covariate, and also their interaction term. We then found the MAM as before. We also tested here whether foundress clutch size was a better predictor of clutch composition than log oviposition bout length. We added the term to the male production, female production and clutch sex ratio MAMs (log transformed following visual inspection of the data in the male production and clutch sex ratio analyses), then tested its significance and that of log bout length by removing them in turn from the model. Clutch size was positively correlated with bout length (see §3), so a potential problem was that the two variables were so collinear that they cancelled out each other in the statistical model (Crawley 2002). However, in each of our analyses, we found that when clutch size was added, the effects of oviposition bout length (and foundress type when it was also significant) were no longer significant, i.e. clutch size was as good, if not better, predictor of clutch composition (for these analyses, we also give the percentage of deviance explained by the two MAMs). All analyses were carried out in S-PLUS 7.06 Professional Edition (Insightful Corp 2007).

3. RESULTS

(a) Brood size and composition of winged and wingless foundresses

Table 1 summarizes brood size and composition in figs oviposited in by winged foundresses entering their first fig and wingless foundresses entering their second fig. The relationship between foundress density and brood size differed between foundress types (LM: foundress type $\times$ density interaction $F_{2, 152} = 5.11$, $p < 0.01$; see also figure 1a). Brood size in winged foundress broods decreased with foundress density (LM on winged foundress data only: $F_{2, 128} = 5.33$, $p < 0.01$): single-foundress

Figure 1. Relationships between (a) foundress density and brood size and (b) foundress density and brood sex ratio, in broods containing the offspring of winged foundresses depositing their first clutches (filled bars) or wingless foundresses depositing their second clutches (open bars). Mean values are shown $\pm$ s.e.

broods were larger ($\beta \pm \text{s.e.} = 12.32 \pm 3.79$, $F_{1, 129} = 10.56$, $p < 0.01$) than two- or three-foundress broods (which did not differ: $F_{1, 128} = 0.17$, n.s.). By contrast, in wingless foundresses, a positive trend was observed (LM on winged foundress data only: $F_{2, 24} = 3.00$, $p = 0.07$): three-foundress broods were larger ($\beta \pm \text{s.e.} = 19.16 \pm 8.49$, $F_{1, 25} = 5.09$, $p < 0.05$) than single- and two-foundress broods (which did not differ: $F_{1, 24} = 0.92$, n.s.). Single wingless foundress brood size was approximately 50% of that of single winged foundresses, but given increases in wingless foundress brood size with density in three-foundress broods the two-foundress types produced similar-sized broods. Hence, wingless foundresses were less oviposition site limited than winged foundresses.

The relationship between foundress density and brood sex ratio also differed between foundress types (GLM: foundress type $\times$ density interaction $F_{2, 152} = 6.32$, $p < 0.01$; see also figure 1b). Winged foundress brood sex ratios increased with increasing foundress density (LM on winged foundress data only: foundress density $F_{2, 128} = 49.90$, $p < 0.001$; single versus two foundresses $\beta \pm \text{s.e.} = 0.89 \pm 0.09$, $F_{1, 128} = 47.98$, $p < 0.001$; two versus three foundresses $\beta = 0.27 \pm 0.08$, $F_{1, 128} = 4.80$, $p < 0.05$). By contrast, wingless foundress brood sex ratios were independent of density (LM on wingless foundress data only: $F_{2, 24} = 0.73$, n.s.). Wingless foundress brood sex ratios were less female biased than those of winged foundresses in single- and two-foundress broods, but were similarly biased in three-foundress broods. These sex ratio differences between foundress types were not caused by differences in the number of males produced, which increased with increasing foundress density in both winged and wingless foundress broods (GLM: $\chi^2_2 = 131.22$, $p < 0.001$; single versus two foundresses $\beta \pm \text{s.e.} = 0.47 \pm 0.07$, $\chi^2_1 = 40.51$, $p < 0.001$; two versus three foundresses $\beta = 0.22 \pm 0.07$, $\chi^2_1 = 22.84$, $p < 0.001$), and was slightly higher for wingless foundresses ($\beta = 0.18 \pm$

0.07 , $\chi^2_1 = 6.48$, $p < 0.05$; foundress type $\times$ density interaction $\chi^2_2 = 4.12$, n.s.; see also table 1). Rather, they were due to patterns of female production. The relationship with foundress density differed between foundress types (GLM: foundress type $\times$ density interaction $F_{2, 152} = 5.76$, $p < 0.01$; see also table 1). Winged foundress female production decreased with foundress density (GLM on winged foundress data only: $F_{2, 128} = 11.17$, $p < 0.001$): it was greater in single-foundress broods ($\beta \pm \text{s.e.} = 16.95 \pm 3.57$, $F_{1, 129} = 22.50$, $p < 0.001$) than in two- or three-foundress broods (which did not differ: $F_{1, 128} = 0.01$, n.s.). By contrast, wingless foundress production increased non-significantly with density (GLM on wingless foundress data only: $F_{2, 25} = 1.92$, n.s.). Wingless foundresses produced fewer females than winged foundresses in single- and two-foundress broods, but in three-foundress broods the two-foundress types produced similar numbers of females.

(b) Clutch size and composition with differing oviposition bout length

Table 2 summarizes the clutch size and composition of winged foundresses and wingless foundresses allowed to oviposit for different lengths of time before the bout was terminated. Both winged and wingless foundresses had high initial rates of offspring production, averaging over one offspring per minute in the first 5 min after entry. Following this, oviposition rates declined progressively at a rate that was quicker in wingless foundresses: their clutch sizes increased at a much lower rate for given bout length than those of winged foundresses (GLM: foundress type $\times$ log bout length interaction; $\beta \pm \text{s.e.} = -0.23 \pm 0.04$, $\chi^2_1 = 43.41$, $p < 0.001$; figure 2a).

Approximately half of the male offspring produced were deposited in the first 5 min of oviposition bouts, with small numbers continuing to be produced later in bouts (table 2; GLM: log bout length; $\beta \pm \text{s.e.} = 0.21 \pm 0.04$, $\chi^2_1 = 28.05$, $p < 0.001$). Winged foundress production was slightly but non-significantly higher for a given bout length than that of wingless foundresses ($\chi^2_1 = 0.99$, n.s.; foundress type $\times$ log bout length interaction $\chi^2_1 = 1.93$, n.s.; deviance explained by MAM = 36%). When foundress clutch size was added to the statistical model, though, it better predicted male production than bout length: a positive logarithmic relationship was found ($\beta = 0.47 \pm 0.08$, $\chi^2_1 = 38.59$, $p < 0.001$; figure 2b), with log bout length no longer significant ($\chi^2_1 = 0.01$, n.s.; foundress type $\chi^2_1 = 1.73$, n.s.; foundress type $\times$ log bout length interaction $\chi^2_1 = 0.01$, n.s.; deviance explained by MAM = 50%).

Female offspring production also increased at a decreasing rate with oviposition bout length (table 2). However, this time the relationship differed between foundress types (GLM: foundress type $\times$ log bout length interaction; $\chi^2_1 = 37.07$, $p < 0.001$): winged foundress production increased at a higher rate ($\beta \pm \text{s.e.} = 0.55 \pm 0.02$) than wingless foundress production ($\beta = 0.31 \pm 0.04$; deviance explained by MAM = 86%). As with male production, though, female production was best predicted by foundress clutch size. When the term was added to the statistical model, a linear positive relationship was found ($\beta = 0.03 \pm 0.0001$, $\chi^2_1 = 1076.79$, $p < 0.001$; figure 2c), with foundress type ($\chi^2_1 = 0.59$, n.s.), log bout length ($\chi^2_1 = 0.03$, n.s.) and their interaction ($\chi^2_1 = 0.67$, n.s.) no longer significant (deviance explained by MAM = 96%).

Table 2. Clutch size and composition for single winged and wingless *L. tentacularis* foundresses ovipositing for set periods of time (see §2 for sample sizes).

	oviposition time (min)	winged foundresses		wingless foundresses	
		mean $\pm$ s.e.	range	mean $\pm$ s.e.	range
clutch size	5	7.10 $\pm$ 1.34	5–19	6.44 $\pm$ 0.44	5–8
	15	15.45 $\pm$ 0.77	11–20	13.00 $\pm$ 0.65	11–16
	30	19.18 $\pm$ 1.16	13–25	16.60 $\pm$ 0.85	13–20
	60	28.46 $\pm$ 1.72	19–41	18.20 $\pm$ 1.31	11–24
	120	38.36 $\pm$ 3.17	19–54	19.99 $\pm$ 1.09	15–25
	240	54.47 $\pm$ 2.49	34–73	27.80 $\pm$ 1.29	21–38
males produced	5	0.15 $\pm$ 1.70	1–2	2.00 $\pm$ 0.23	1–3
	15	2.63 $\pm$ 0.30	1–5	2.75 $\pm$ 0.25	2–4
	30	3.00 $\pm$ 0.42	2–6	3.10 $\pm$ 0.45	2–6
	60	3.13 $\pm$ 0.32	2–6	3.33 $\pm$ 1.26	2–5
	120	4.00 $\pm$ 0.33	2–6	3.11 $\pm$ 0.26	2–4
	240	5.00 $\pm$ 0.49	3–11	3.90 $\pm$ 0.26	3–5
females produced	5	1.30 $\pm$ 5.40	3–17	4.44 $\pm$ 0.37	3–6
	15	12.81 $\pm$ 0.73	8–17	10.25 $\pm$ 0.59	8–12
	30	16.18 $\pm$ 0.96	11–21	13.50 $\pm$ 0.61	11–17
	60	25.33 $\pm$ 1.49	17–35	14.90 $\pm$ 1.26	8–21
	120	34.36 $\pm$ 2.96	17–50	16.68 $\pm$ 1.02	12–21
	240	49.47 $\pm$ 2.37	31–65	20.60 $\pm$ 1.24	15–27
clutch sex ratio	5	0.31 $\pm$ 0.27	0.11–0.40	0.31 $\pm$ 0.03	0.20–0.50
	15	0.17 $\pm$ 0.01	0.06–0.27	0.21 $\pm$ 0.01	0.14–0.27
	30	0.15 $\pm$ 0.01	0.10–0.26	0.18 $\pm$ 0.02	0.11–0.30
	60	0.11 $\pm$ 0.01	0.06–0.15	0.18 $\pm$ 0.01	0.13–0.27
	120	0.10 $\pm$ 0.01	0.07–0.15	0.16 $\pm$ 0.01	0.10–0.20
	240	0.09 $\pm$ 0.01	0.06–0.22	0.16 $\pm$ 0.01	0.13–0.21

Thus, differences between foundress types for a given bout length arose because wingless foundresses deposited smaller clutches.

As might be expected, clutch sex ratios reflected these production patterns (and their causes). They decreased at a decreasing rate with oviposition bout length (table 2; GLM: log bout length; $\beta \pm$ s.e. -0.26 ± 0.05 , $\chi^2_1 = 28.72$, $p < 0.001$) and were more male biased (higher) in wingless foundresses than winged foundresses ($\beta = 0.43 \pm 0.11$, $\chi^2_1 = 14.68$, $p < 0.001$; foundress type $\times$ log bout length interaction; $\chi^2_1 = 1.70$, n.s.; deviance explained by MAM = 53%). Similar to the male and female production data, though, clutch sex ratio was best predicted by foundress clutch size. When the term was added to the statistical model, a negative logarithmic relationship was found (log clutch size: $\beta = 0.63 \pm 0.09$, $\chi^2_1 = 53.57$, $p < 0.001$; figure 2d), and the effects of log oviposition bout length ($\chi^2_1 = 0.01$, n.s.) and foundress type ($\chi^2_1 = 1.81$, n.s.) were no longer significant (foundress type $\times$ log bout length interaction $\chi^2_1 = 0.02$, n.s.; deviance explained by MAM = 54%).

4. DISCUSSION

Our results experimentally confirm that *L. tentacularis* fig wasp females (foundresses) use their clutch size as a cue to assess potential LMC (Hamilton 1967) when determining the sex ratio of the offspring they deposit in figs. We found that wingless foundresses depositing their second clutches produced smaller clutches than winged foundresses that were depositing their first clutches, and so were less oviposition site limited in figs containing other similar foundresses (foundresses deposit single eggs in female fig flowers, so deposition

is ultimately limited by the number of flowers in the fig). Consequently, we also found that wingless foundress whole fig (brood) sex ratios independence of foundress density is consistent with the increases in winged foundress brood sex ratios with density (see also Moore *et al.* 2002) arising only because oviposition site limitation in multi-winged foundress figs leads to smaller more male-biased clutches, and not because of to any direct response to foundress density. Additionally, using a new technique that allowed us to terminate oviposition bouts at different points in time, we found that foundresses deposited mostly male offspring at the beginning of oviposition bouts, but later on deposited mostly female offspring with only a few males. This is consistent with the predictions of a theoretical model previously developed concerning optimal sex ratios when foundresses adjust their strategy according to clutch size (Moore *et al.* 2005), rather than with another strategy that could explain the patterns observed: that they deposit a set number of male offspring, followed by only females (see also Herre *et al.* 1997; Moore *et al.* 2002; Kjellberg *et al.* 2005).

These findings are a rare demonstration of the cues used by foundresses to assess potential LMC when determining their sex ratio (for another example, see Shuker & West 2004). The determination of cue use patterns in such species is of general importance to evolutionary biologists because of its implications for our understanding of whether there are constraints upon the precision of adaptation (see also Herre 1987; West & Herre 1998; Herre *et al.* 2001). Deviations in fit from the quantitative predictions of classical LMC theory (see Hamilton 1967) have been hypothesized to be a consequence of foundresses using cues other than

Figure 2. Relationships between (a) oviposition bout length and foundress clutch size, (b) foundress clutch size and male offspring production, (c) foundress clutch size and female offspring production and (d) foundress clutch size and clutch sex ratio during the oviposition bout termination experiments. Squares indicate winged foundresses depositing their first clutch and circles indicate wingless foundresses depositing their second clutch.

foundress density (as in the classical models) to assess potential LMC, rather than limits to the ability of natural selection to produce perfect organisms (West *et al.* 2000, 2002; West & Sheldon 2002; Moore *et al.* 2005; for discussion of factors likely to constrain adaptation, see Herre 1987; West & Herre 1998; Herre *et al.* 2001). Our findings support this hypothesis: they experimentally confirm that deviations from classical theory predictions in *L. tentacularis* (more female-biased brood sex ratios than expected at each foundress density; Moore *et al.* 2002) occur because foundresses use clutch size as a cue to assess potential LMC (see also Moore *et al.* 2005). Of course, with regard to whether adaptation is constrained, this means the question becomes how closely foundress strategies approximate theoretical optima for a given clutch size. As noted previously, *L. tentacularis* foundress clutch sex ratios closely approximate the quantitative predictions of relevant theory (Moore *et al.* 2005), so it would appear that in this species selection produces perfect sex ratios, i.e. its action is unconstrained. Further work investigating foundress cue use patterns and the fit of the sex ratios produced to relevant theoretical predictions (see also below) is now required to test whether this is also true in other species in which deviations from the predictions of classical LMC theory occur (see also Molbo *et al.* 2003, 2004).

Our findings also have several other implications. One of these is that they raise the question of how foundresses assess clutch size. Inferences concerning this are possible from our analyses of the factors determining clutch composition and sex ratio during the oviposition sequence experiments. These indicate that differences in clutch composition/sex ratio over bouts (clutch sex ratios became more female biased as bouts continued, because foundresses deposited relative more female offspring), and indeed between winged and wingless foundresses (the latter produced smaller clutches with fewer female

offspring for given bout length, and so less female-biased sex ratios), can be explained solely by differences (changes) in clutch size. This is inconsistent with the use of the most likely method of indirectly assessing clutch size, the rate at which eggs are deposited, which would be predicted to lead to additional positive effects of bout length on clutch sex ratio even after clutch size is taken into account (similar would also be expected with regard to wingless versus winged foundresses, as the former oviposit more slowly). Hence, a more direct method of assessment is probable. One possibility is that foundresses use information from physiological processes related to the number of eggs yet to be deposited (their 'egg load'), a cue hypothesized to be important in many aspects of insect oviposition behaviour (see Minkenberg *et al.* 1992; Hempel *et al.* 1998). If this is true in *L. tentacularis*, though, foundresses must also be able to 'reset the clock' after their first clutch: they begin their second clutch by depositing mostly male eggs again (see Gibernau *et al.* 1996 for indirect evidence of similar behaviour in the fig wasp *Blastophaga psenes*).

The second implication of our findings concerns why *L. tentacularis* foundresses use clutch size to assess potential LMC. Such cue use is apparently not universal in fig wasps: clutch sex ratios imply that *Blastophaga nipponica* foundresses also use foundress density (Kinoshita *et al.* 2002; see Moore *et al.* 2005 for discussion of cue use patterns in non-fig wasp species). We have previously argued that the cue use pattern in *L. tentacularis* occurs because increasing oviposition site limitation in multi-foundress figs means that clutch size supplies sufficiently accurate information about potential LMC that the use of foundress density as a cue has not evolved, and also that when oviposition site limitation in multi-foundress figs is less severe, the information supplied by clutch size will be less accurate and so the use of foundress density will evolve as well (Moore *et al.* 2005).

These arguments receive support from *B. nipponica*: foundresses are less oviposition site limited in multi-foundress figs than in *L. tentacularis* (Kinoshita et al. 2002). The findings presented here, though, indicate another factor likely to affect cue use patterns. *L. tentacularis* foundress first (winged) and second (wingless) clutches differ in size (see Gibernau et al. 1996 for similar in *B. psenes*). The extent to which other foundresses depositing offspring affects potential LMC depends on their relative contribution to the brood (Frank 1985; Stubblefield & Seger 1990; Moore et al. 2005), so this will reduce the accuracy of the information foundress density supplies about potential LMC and therefore affect whether its use as a cue evolves. We also note here that the accuracy of foundress density as a cue is likely to be reduced by sequential entry to figs in the field: this will lead to unequal brood contributions if oviposition site limitation occurs in multi-foundress figs, and, assuming physical interaction is required, underestimation of actual foundress density if re-emergences occur (see Greeff & Compton 1996; Kathuria et al. 1999; Kinoshita et al. 2002; Moore et al. 2002). Consequently, in fig wasps at least clutch size may often supply more accurate information about potential LMC than foundress density and therefore be more likely to be used as a cue (see also Frank 1985; Stubblefield & Seger 1990). Further research investigating this adaptive interpretation of cue use in fig wasps (and other species) is now required. Theory should compare strategy fitnesses under different cue use patterns to give explicit predictions about when they will evolve. Empirical work should determine population parameters and cue use patterns in other species, so models can be parametrized and predictions tested. For this latter work, the techniques introduced in this paper should prove invaluable.

In conclusion, we have shown experimentally that *L. tentacularis* fig wasp foundresses use their clutch size as a cue to assess potential LMC when determining the sex ratio of offspring deposited. This confirms that deviations from the quantitative predictions of classical LMC theory in the species occur because cues other than foundress density are used to assess potential LMC, and not because of constraints upon the precision of adaptation. We then discuss how foundresses assess clutch size and why such a cue use pattern is employed. We end by calling for similar work on other species: this will allow the generality of our conclusions concerning the ability of natural selection to produce 'perfect' organisms to be evaluated, and hypotheses concerning adaptive variation in the cues used to assess environmental conditions to be tested.

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