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The presence of a guard vicariously drives split sex ratios in a facultatively social bee

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Split sex ratios provide broad insights into how reproductive strategies evolve, and historically have special relevance to the evolution of eusociality. Yet almost no attention has been directed to situations where split sex ratios may potentially decrease the payoffs for worker-like behaviour, increasing selective thresholds for eusociality. We examined sex ratios in a facultatively social colletid bee, *Amphylaeus morosus*. Sex ratios in this bee vary strongly with the presence of a nest guard and in a pattern that does not conform to assumptions of previous models in which split sex ratios facilitate altruism. While the production of daughters was constant across social and solitary nests, mothers produced more brood when a non-reproductive guard was present, but these extra brood were all male. This leads to split sex ratios, vicariously driven by guards that are unable to manipulate sex ratios in their favour. Importantly, if guarding becomes more common in a population this would lead to an excess of males and lower the genetic value of these extra males to guards, effectively putting a brake on selection for worker-like behaviour.

1. Introduction

Fisherian principles state that under most conditions, natural selection should favour equal investment in sons and daughters, leading to balanced population investment ratios [1]. At the same time, individual parents should bias their offspring towards the sex that will generate the greatest return on their individual investments [2]. In haplodiploid populations, males develop from an unfertilized egg, such that mated females can control the sex of their offspring during oviposition; this flexibility allows mothers to skew sex allocation ratios in response to ecological, social or physiological cues [3]. In some cases, biased ratios can arise under conditions that drive individual deviations from a Fisherian parity [4]. Split sex ratios—where colonies specialize in either gyne or male production—can provide broad insights into how reproductive strategies evolve, in particular the evolution of workers in eusocial colonies.

Inclusive fitness theory predicts that female workers in eusocial hymenopteran societies should favour rearing sisters over brothers because haplodiploidy results in a much higher relatedness to the former ($r = 3/4$) than the latter ($r = 1/4$) [5,6]. By contrast, queens should generally prefer to invest equally in daughters and sons because of Fisherian dynamics [1,7], and this difference in preferred sex allocation strategies can lead to queen–worker conflict [3,8]. In populations containing both solitary and social colonies, split sex ratios may help facilitate the evolution of eusociality [9,10]. In the facultatively social bee, *Megalopta genalis*, sex ratios are biased in accordance with the inclusive fitness predictions for worker control, with female-biased brood in social nests favouring worker-controlled investment. In this case-study, workers were able to access provisioned brood cells during the rearing phase, but were never directly observed to manipulate sex ratios

Table 1. Split sex ratios and productivity of *Amphylaeus morosus* solitary and social colonies. Ninety-five per cent confidence intervals for mean NSR are bootstrapped over 2000 pseudo-replicates. Values presented as mean ± s.e. and NSR presented as M : F.

	nest type	sample size (nests)	mean clutch size	mean pupal weight (mg)			mean NSR		
				female	male	arithmetic	95% CI	arcsine back transformed	95% CI
social status	solitary	180	5.14 ± 0.219	61.15 ± 0.669	54.19 ± 0.791	0.338	0.282–0.395	0.241	0.174–0.316
	social	13	12.2 ± 0.725	58.15 ± 2.99	57.62 ± 1.39	0.834	0.785–0.879	0.850	0.791–0.905
population	mixed	193	5.49 ± 0.229	61.31 ± 0.65	55.05 ± 0.69	0.568	0.536–0.599	0.606	0.557–653

[10]. Sex-biased ratios have been shown to sometimes lower the benefits needed for worker altruism to persist under haplodiploidy, but whether this mechanism is strong enough to facilitate the evolution of eusociality is contested [9,11,12].

Many studies have argued that female-biased sex ratios can lower thresholds for sib-rearing [13,14]. However, few have explored whether split sex ratios can act to raise the thresholds for worker altruism to become a widespread strategy. We use sex ratio data for the only known social species in the bee family Colletidae, *Amphylaeus morosus* (Smith, 1879) (Hymenoptera: Colletidae), to show how male-biased sex ratios in social colonies challenge common assumptions of split sex ratio evolution in social Hymenoptera. Social nesting in *A. morosus* is facultative and relatively rare, with approximately 4.3% of nests containing two or more adult females [15]. Colonies generally comprise either matrifilial or sororal pairings [16]. In these colonies, reproduction is monopolized by one female that forages and provisions her own brood while the non-reproductive, guard female defends the nest from invaders. Despite the monopoly over reproduction in social nests, guards are always mated and able to disperse and reproduce as solitary foundresses throughout the reproductive season [16]. Here we show that the presence of a nest guard leads to split sex ratios in a way that heightens, rather than lowers, the selective threshold for eusociality.

2. Methods

Nests of *A. morosus* ($n = 298$) were collected from the Dandenong Ranges, Victoria, Australia throughout the reproductive season. Nests were sampled across 5 consecutive years (2017–2021; equating to four reproductive seasons) and seven separate collections (electronic supplementary material, appendix).

To assess patterns of investment sex ratios (ISRs) in colonies of *A. morosus*, brood sex, wet weight and brood cell position were recorded. Pupae were weighed on a Thermoline precision balance to ±0.1 mg. The numerical sex ratio (NSR) was calculated as the number of male brood divided by the total number of brood that reached pupation to a point that they could be reliably sexed ($\text{NSR} = \Sigma_{\text{male brood}} / \Sigma_{\text{male brood + female brood}}$). ISR was calculated as a product of the NSR and the pupal weight ratio calculated from mean brood sex pupal weight and was used to test whether the observed NSR deviated from the investment a mother allocates to each sex. Brood that had died before nests were opened were not included in pupal weight measurements but were used for NSR calculations. To examine sex allocation patterns across cell positions in the nest, cell position was coded so that ‘cell 1’ corresponded to the first cell provisioned (furthest from the nest entrance), following Hearn *et al.* [17]. For some analyses, brood that reached adulthood were pooled across all nests and the pupal sex was treated as a binomial response variable (female = 0, male = 1). For all analyses, we define solitary nests as nests containing one or no adult female at the time of collection and social nests as colonies containing two adult females or genome-inferred social nests (see Hearn *et al.* [16]). However, social nesting is likely to be more common than our nest census data suggest (electronic supplementary material, appendix).

All statistical analyses were performed in [18] and [19]. Tests of normality and homoscedasticity were assessed using a Shapiro–Wilk test and Levene’s test. A chi-square goodness of fit test was used to determine if observed NSR significantly differed from an expected null hypothesis of equal investment. Where necessary we used arcsine transformed values of sex ratio and the corresponding confidence intervals. Unless otherwise stated, values are presented as mean ± s.e.

3. Results

(a) Population-wide sex allocation

We collected 193 viable *A. morosus* nests containing 392 female brood and 516 male brood in total. On average, female pupae were 1.11 times heavier than males (female brood: 61.31 ± 0.65 mg; male brood: 55.05 ± 0.69 mg; independent samples *t*-test: $F_{858} = 15.926$, $p < 0.001$). The ISR was slightly male biased and significantly differed from an expected 1 : 1.11 female : male ratio ($\text{ISR} = 0.523$, $n = 908$). Mean pupal weight for each sex did not differ between solitary and social colonies (female brood: independent samples *t*-test: $F_{363} = 3.640$, $p = 0.057$; male brood: independent samples *t*-test: $F_{493} = 0.008$, $p = 0.929$; table 1).

(b) Sex ratio across cell position

The population numerical ratio showed a positively increasing sigmoidal trend as cell position increased (figure 1). In highly productive nests (greater than seven brood cells), female brood occurred primarily in the first seven cells (cell positions 1–7; $\text{NSR} = 0.249$, $\chi^2 = 100.50$, $p < 0.001$), whereas the numerical ratio of the later cells was entirely male-biased (cell positions 8–17; NSR = 1.0; table 2).

(c) Split sex ratios

Nests of *A. morosus* showed split sex ratios (table 1). The NSR was significantly associated with social status ($r_s = 0.305$, $p < 0.001$), where social nests were heavily male biased (social NSR = 0.850), while the mean sex ratio for solitary nests was female-biased (solitary NSR = 0.338; table 1). However,

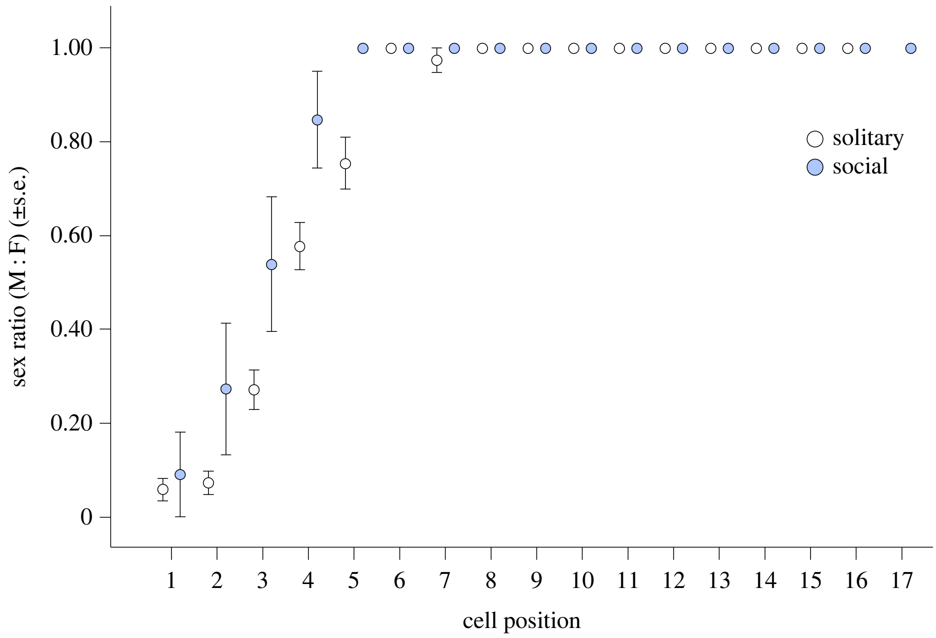

Figure 1. The comparison of sex ratios across nest cell positions for solitary and social nests of *Amphylaeus morosus*. Error bars are presented as 95% confidence intervals estimated from 2000 bootstrap pseudo-replicates. Sex ratio is presented as the proportion of males.

Table 2. Sex allocation of *Amphylaeus morosus* offspring across cell positions for solitary and social colonies. Deviations from a hypothesized 0.5 sex ratio are shown with a chi-square goodness of fit test. Values presented as mean ± s.e. and NSR presented as M : F.

	solitary					social				
brood cell position	total no. of brood	no. of females	offspring NSR	χ^2	*p*-value	total no. of brood	no. of females	offspring NSR	χ^2	*p*-value
1	119	112	0.058 ± 0.022	92.65	<0.001	11	10	0.091 ± 0.091	7.36	0.007
2	123	114	0.073 ± 0.024	89.63	<0.001	11	8	0.273 ± 0.141	2.27	0.132
3	114	83	0.272 ± 0.042	23.72	<0.001	13	6	0.539 ± 0.142	0.08	0.782
4	97	41	0.577 ± 0.050	2.32	0.128	13	2	0.846 ± 0.104	6.23	0.013
5	61	14	0.754 ± 0.056	15.75	<0.001	13	0	1.00	—	—
6	39	0	1.00	—	—	14	0	1.00	—	—
7	38	1	0.974 ± 0.026	34.11	<0.001	12	0	1.00	—	—
8–17	167	0	1.00	—	—	63	0	1.00	—	—

the mean number of female offspring was not significantly different between solitary and social nests (mean female offspring: solitary = 1.96 ± 0.09, social = 1.86 ± 0.29; Mann–Whitney test: $U = 1235.0$, $p = 0.872$), but the number of male offspring was significantly greater in social nests (mean male offspring: solitary = 2.16 ± 0.25, social = 9.00 ± 0.71; Mann–Whitney test: $U = 207.5$, $p < 0.001$; figure 2).

We examined whether sex ratio variation was explained by total brood size or social/solitary status using an ANCOVA with social status as the treatment and total brood size as the covariate. Sex ratio was arcsin transformed because it is a zero/one truncated variable. Our analysis indicated no effect of social status ($F_{1,197} = 0.217$, $p = 0.642$), but a highly significant effect of brood size ($F_{1,197} = 114.023$, $p < 0.001$). This indicates that the impact of guards on sex ratios is due to their positive effect on brood size, *per se*, rather than queens adjusting sex ratios because of simply having a helper.

4. Discussion

Split sex ratios have been widely discussed as a feature that may facilitate the evolution of eusociality in haplodiploids, but such conclusions are largely based on the assumption that worker presence within colonies is associated with female-biased colony-specific sex ratios. Our studies on *A. morosus* reveal a pattern that is opposite to common assumptions for split sex ratios in social Hymenoptera.

Sex allocation patterns in *A. morosus* are strongly impacted by the presence of a nest guard, even though guards are unable to directly control sex allocation. Instead, having a guard allows mothers in social nests to provision extra offspring, but those additional brood are males and their relatedness to guards would be mostly as brothers ($r = 1/4$) or nephews ($r = 3/8$). In some social Hymenoptera, sex ratios have been shown to vary in response to whether workers were sisters or

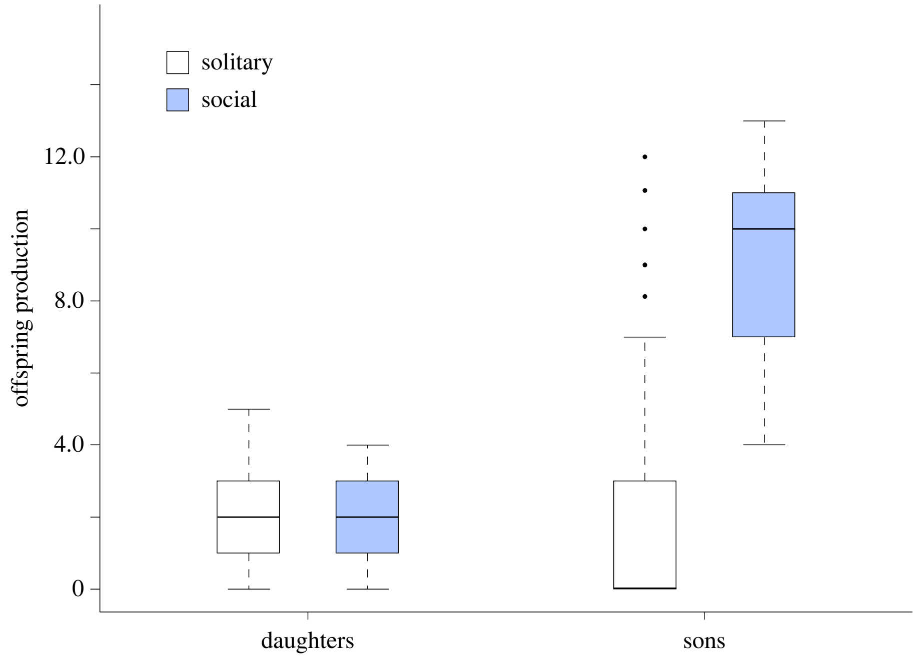

Figure 2. Box plots showing the constant production of daughters and the skewed production of sons across both solitary and social *Amphylaeus morosus* nests. Box plots represent the median, interquartile range and range of the data.

aunts to the queen's offspring [20,21]. By contrast, for *A. morosus*, mean sex ratios did not differ between matrifilial and sororal colonies [16]. The difference in indirect fitness gains to guards are therefore lower than if sex ratios were female-biased, where guards would be primarily aiding sisters ($r = 3/4$) or nieces ($r = 3/8$).

Importantly, if the frequency of guarding in a population were to increase, the resulting over-production of males would devalue their genetic worth to guards. This is similar to the argument famously raised by Trivers & Hare [3] that worker-mediated overinvestment in female brood should stabilize at a 1 : 3 (M : F) sex ratio where the genetic value of brothers and sisters to workers reaches parity. In *A. morosus*, guards are unable to directly control the sex of brood, so their ability to influence population-wide sex ratios is simply one of assuming a guarding role or else nesting solitarily. This should lead to frequency-dependent selection on guarding behaviour *per se*: as guarding becomes more common, the value of the resulting additional males would become lower, putting a brake on the frequency of guarding behaviour.

The sequential production of daughters followed by sons in *A. morosus* could be explained by a combination of extrinsic and developmental factors. Females are larger than males and take longer to develop from egg to adult (L.R.H. pers. obs.). *Amphylaeus morosus* mothers might therefore produce the sex with the longest development time first to avoid subsequent brood cells being disrupted as earlier brood mature [22]. Another factor may involve seasonal resource availability. For example, if floral resources are abundant early in the season, mothers should initially invest in the more costly sex (i.e. females) and then switch to the less costly sex (i.e. males) to ensure the greatest return on investment [15,23–25]. This pattern suggests that sperm depletion could factor into the switch to male brood production from females. However, a previous study showed that all mated queens of *A. morosus* had sperm in their spermatheca after egg-laying had finished [16]. Rather, this pattern concords strongly with the 'constant philopater' hypothesis (CPH; [26]). CPH predicts that a constant number of the more-philopatric sex, in our case females, which re-use their natal nest 58% of the time (electronic supplementary material, appendix), are produced regardless of maternal condition or the total number of brood, to reduce competition between philopatric kin and to avoid underinvestment if resources later become scarce [26].

We propose that *A. morosus* exhibits a form of social evolution that acts as an impediment to eusociality and directly contrasts with how split sex ratios have usually been thought to affect worker evolution [12,27]. Male-biased broods in social colonies arise from the benefit of having a designated nest guard that allows provisioning mothers to increase their offspring production, but where producing more of the larger sex (females) is likely to be constrained. However, this increase in male offspring production reduces the payoffs for guarding behaviour. As guards become more common in a population, the reproductive value of males declines as the number of males increases. This effect may put a 'selective brake' on the frequency of females opting to take on guarding roles and limit the potential for guarding behaviour to spread. This might help explain why guarding behaviour in *A. morosus* is consistent with inclusive fitness theory but is nevertheless uncommon [16].

Data accessibility. The analyses reported in this article can be reproduced using the data provided in Hearn *et al.* [28].

The data are provided in the electronic supplementary material [29].

Authors' contributions. L.R.H.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, validation, visualization, writing—original draft and writing—review and editing; M.I.S.: conceptualization and writing—review and editing; M.P.S.: conceptualization, formal analysis, methodology, writing—original draft and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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