



Nesting biology and social organisation of a silk wasp (*Microstigmus rosae*) from the North–West Ecuadorian Choco

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Abstract

Microstigmus Ducke (Hymenoptera, Apoidea, Crabronidae, Pemphredoninae) is an intriguing genus of wasp, particularly interesting for the study of social evolution as it contains the only species of apoid wasps known to exhibit eusocial behaviours. Thus far, data concerning the genetics and biology of sociality in *Microstigmus* are restricted to just two species. Through behavioural observations and genetic analyses, we here present a detailed report on the nesting biology and social organisation of a third species, *Microstigmus rosae*. Our results indicate that *M. rosae* nests are founded by a single female and social groups appear when emerging female offspring remain on the natal nests as helpers. Adult females mass provision their offspring and developmental data are consistent with sequential brood production, an average egg laying interval of 10 days and a brood developmental period of more than 35 days. Social group formation is atypical, with 20% of nests containing up to a maximum of four adult females and two males. Adult group size is positively correlated with the number of brood in a nest, but negatively correlated with per capita brood. The sex investment ratio was female biased. Our findings support several theories concerning the behavioural characteristics thought to be important for transitions towards sociality, namely: high genetic relatedness generated by monogamous matrifilial associations, hygienic behaviour and the potential for direct reproduction. In contrast, one hypothesised trait, progressive provisioning, is notably absent, highlighting the need for investigations into the significance of post-provisioning parental care for offspring survival in this genus.

Keywords *Microstigmus* · Nesting biology · Sociality · Parental care · Reproductive skew

Introduction

Sociality is ubiquitous in nature and has evolved multiple times throughout the animal kingdom (Wilson 1971). The result is a diverse range of social lifestyles, varying from simple cooperatively breeding groups comprising totipotent individuals to advanced eusocial societies with morphologically distinct queen and worker castes that are irreversibly committed to their roles (Wilson 1971; Crespi and Yanega 1995). Social behaviour is of particular interest to the field of evolutionary biology as it embodies what is thought to underlie many of the key transitions in the evolution of complex life: the progression from solitary, independent

reproduction to coordinated groups cooperating in reproductive activities (Maynard Smith and Szathmary 1997; West et al. 2015). However, the apparently selfless behaviour fundamental to eusocial societies presents an evolutionary paradox: How can natural selection, traditionally considered to operate through enhanced personal fitness, produce individuals which help others at a cost to their own personal reproductive output?

Though many different explanations have been put forward to explain transitions towards sociality, to date the most widely accepted theoretical framework is Hamilton's concept of inclusive fitness (Hamilton 1963, 1964; Bourke 2011). Hamilton's insight that individuals can gain fitness not only through their own personal reproductive output but also via their impact on the reproduction of relatives has become a cornerstone of social evolutionary theory. A simple, yet highly influential parameterisation of these ideas is provided by Hamilton's Rule which states that genes for any cooperative behaviour will be favoured by selection when the sum of indirect fitness (rb) and direct fitness (c) exceeds

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zero, where c is the fitness cost to the altruistic actor, b is the fitness benefit to the recipient, and r is their genetic relatedness (Hamilton 1964; Charnov 1977; Grafen 1982). It thereby follows that life history characteristics influencing either relatedness or the likely costs and benefits of social behaviour will be critical for the evolution of sociality in natural populations.

Of the proposed prerequisites to social evolution, high genetic relatedness is the most widely recognised. By aiding the reproduction of closely related individuals, helpers will indirectly contribute to the transmission of their own genes to the next generation (Hamilton 1964; Bourke 2014). Thus, life-history characteristics thought to increase genetic relatedness between helping individuals, such as lifetime monogamy (Hughes et al. 2008; Boomsma 2009; Cornwallis et al. 2010; Lukas and Clutton-Brock 2012; Smith et al. 2018), inbreeding (Wade and Breden 1981; Breden and Wade 1981; Chapman et al. 2000) and haplodiploid sex determination (Hamilton 1964; West-Eberhard 1975), are commonly cited as likely preconditions for the evolution of eusociality (Bourke 2011). However, while high relatedness between group members is undoubtedly critical to the evolution of eusociality in many species, the question of why this behaviour has evolved in some lineages but not others remains largely unresolved. Other proposed pre-adaptations concern the ‘ b ’ and ‘ c ’ terms in Hamilton’s rule and include aspects of parental care behaviour such as progressive provisioning of young (Gadagkar 1990; Queller 1994; Field 2005; Linksvayer 2010), opportunities for direct reproduction including the inheritance of valuable nest structures (Piper et al. 1995; Sloane 1996; Shreeves and Field 2002; Leadbeater et al. 2011) and ecological factors such as “harsh” or unpredictable environments (Emlen 1982; Gadagkar 1990; Field et al. 1998; Hatchwell and Komdeur 2000).

One method of exploring the relative importance of these life history characteristics to the spread of sociality is to examine the distribution of these traits in a phylogenetic context (Pagel 1997, 1999). However, although comparative phylogenetic techniques can be effective tools for investigating evolutionary transitions, the power of these analyses is innately restricted by the number of independent transitions towards sociality that exist in nature (Freckleton et al. 2002). Moreover, despite a wealth of data on advanced eusocial groups such as honeybees and ants, life history data for some of the less well-known groups of social Hymenoptera remains a constraint (Linksvayer and Johnson 2019).

Microstigmus Ducke (Hymenoptera, Apoidea, Crabronidae, Pemphredoninae) represents one such group. Unique among the apoid wasps, where solitary behaviour is predominant (Hunt and Toth 2017), species of *Microstigmus* are known to exhibit varying degrees of social complexity (Evans 1966; Matthews 1968; Richards 1972; Ross and Matthews 1989; Melo and Matthews 1997; Lucas et al. 2011b). Restricted to

the neotropical region, this small genus is characterized by a unique method of nest construction whereby organic materials are bound together with silk produced from a gland in the female abdomen (Melo and Matthews 1997). Due to the phylogenetic distance between *Microstigmus* and other eusocial lineages of Hymenoptera (Brothers 1975), sociality in this genus is thought to be the result of convergent evolutionary processes and can therefore provide important contributions to comparative studies of social evolution (Ross and Matthews 1989).

Although some level of primitive or incipiently social behaviour is expected to be occurring in many species of this genus [e.g., *M. thripoctemus*, Asís (2003), *M. similis*, Melo and Evans (1993), *M. myersi*, Melo and Campos (1993), Ross and Matthews (1989)], thus far comprehensive analyses of social structure have only been conducted for two species: *M. comes* and *M. nigrophthalmus*. Both of these species are known to form social groups with reproductive division of labour in egg-laying but no morphological caste differences (Matthews 1968; Lucas et al. 2011b). Despite the potential for important insights for studies of evolutionary transitions towards sociality, *Microstigmus* remains understudied. To develop our understanding of the range of social complexity occurring within *Microstigmus* analyses of additional species are essential.

This paper represents the first analysis of sociality in a newly discovered species, *Microstigmus rosae* Field 2023, which we first located in North–West Ecuador whilst exploring the region in 2014. Through detailed examination of a range of genetic and behavioural characteristics we aim to fulfil two main objectives: (1) to gather an understanding of the social organisation of this species, with reference to social group formation, group size, social structure and reproductive skew, and (2) to present a thorough description of the nesting biology and life history of this new species which can be used for future research. As sociality within *Microstigmus* represents a valuable resource for phylogenetic comparative analyses of social evolution, we place particular emphasis on life history characteristics proposed to be influential to transitions towards social behaviour. Factors include (a) those which influence genetic relatedness between group members, such as female mating frequency, male mating behaviour and inbreeding, and (b) those which determine the costs and benefits of social behaviour such as per capita productivity, opportunities for direct reproduction and aspects of parental care behaviour including brood provisioning strategy, hygienic brood maintenance and defensive behaviour including rates of parasitism.

Methods

Field methods

Nests of *Microstigmus rosae* were collected from two different locations within the Pichincha province of

North-central Ecuador, on the edge of the Andes Mountain range: 1. *Mashpi Biodiversity Reserve*. N 00°10.019' W 078°52.326'; and 2. *Santa Lucia Cloud Forest Reserve*. N 00°07.062' W 078°36.454'. These reserves are made up of a combination of both primary and secondary forests and span an altitudinal range of 750–2300 m above sea level (Fig. 1). Most data were collected during two main field seasons: the first from 31-01-2016 to 01-05-2016 and the second from 12-01-2017 to 10-04-2017. A smaller number of nests were also collected during three preliminary data collection periods which took place from 23-08-2014 to 18-09-2014, from 15-08-2015 to 15-09-2015 and from 01-11-2015 to 21-12-2015.

Microstigmus rosae nests were located by searching the undersides of leaves and other structures. Located nests were categorised into two groups: “newly founded” and “pre-existing”. Newly founded nests were those for which the approximate founding date was known. These nests were identified during the two main field seasons through the systematic monitoring of 987 marked nesting locations. These locations primarily comprised *Xanthosoma sagittifolium* plants, but also included some man-made structures. Once every 5 days, each marked location was examined for both the appearance of newly founded nests

and the continued presence of existing nests. In contrast, pre-existing nests were those nests: (a) located during the three preliminary data collection periods, (b) already established on the marked nesting locations at the start of both main field seasons and (c) found on unmarked nesting locations. Since the founding dates of pre-existing nests were unknown, they represent a “snap-shot” dataset comprising a range of nests at different stages of maturity.

Group size checks were conducted on all accessible pre-existing nests located on marked locations at the start of both main field seasons. The number of adults in nests found in unmarked locations was also checked at the earliest available opportunity, generally the following morning after nest discovery. To perform the group size checks, a small re-sealable transparent plastic bag (13×9 cm) was placed over the nest and, taking care not to cause any damage, the nests were very gently compressed through the bag. This disturbance caused the occupants to leave the nest and become trapped in the bag. Group size checks always took place in the morning before sunrise to ensure that: (a) all nest occupants were likely to be in the nest and (b) adults were able to return to the nest following the disturbance. To collect nests, the same bags were positioned around the nest and the nest petiole was carefully detached from the substrate using

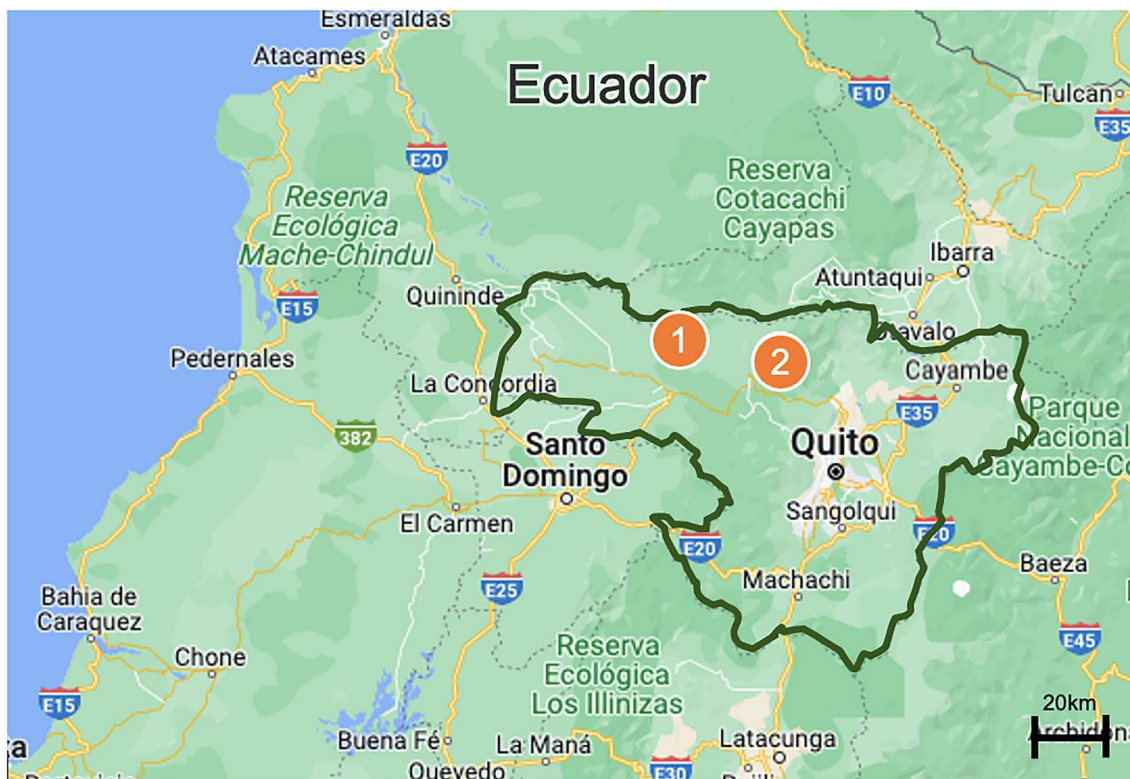


Fig. 1 Map showing the locations of the field sites used for data collection, identified as numbered orange circles: site one is Mashpi Biodiversity Reserve and site two is Santa Lucia Cloud Forest Reserve.

Both field sites are located within the Pichincha province in North-Central Ecuador, outlined in green

forceps and placed into the bag. Nest collection took place in the evening to ensure that all nest occupants were present. Nests were dissected within 24 h of collection using a Leica S6D binocular microscope, adults, prey and brood stages were counted, and the contents stored in 100% ethanol for subsequent genetic analysis. *Microstigmus rosae* individuals were identified and separated from sympatric species using the characteristics described in Field (2023).

Offspring development

The enclosed structure of *M. rosae* nests makes direct observations of brood development impossible. Thus, to build an approximate schedule of offspring development from nest founding to first offspring emergence, we examined data from newly founded nests collected at different ages (0–40 days after nest founding). Using these nests, we were able to estimate the duration of each developmental stage by totalling the average age of nests where the eldest offspring was of each stage, weighted by the proportion of the total number of nests sampled for each nest age group. Nests where the eldest offspring was of a notably less mature developmental stage than other nests in that age group were excluded due to the likely occurrence of brood predation. Nests containing parasitized offspring were also excluded.

Brood developmental stages were categorised in the following manner: eggs are elongate oval, found fixed to an intact prey ball and sealed into a cell. Larvae have a distinct head capsule and were classified as those brood in the process of consuming the prey ball to which they are attached. Once prey ball consumption is complete, the larvae lengthen and assume a distinct elongate form referred to as a prepupa. Pupae can be easily distinguished from the preceding prepupal stage by their resemblance to the adult form. Pupal stages were categorised according to the pigmentation observed in the compound eyes which develop from yellow, to brown and finally black. Where pigmentation on the head, thorax and abdomen was observed, pupae were classified as

pre-adult, the final immature stage. Photographs of several of these brood developmental stages can be seen in Fig. 2.

Genotyping

Genetic structure, genetic relatedness, pedigree relationships and offspring sex were analysed using microsatellite markers. In total, 488 individual adults and brood were genotyped using the microsatellite loci Mni002, Mni003, Mni009, Mni017, Mni019, Mni024, Mni028, Mni030, Mni034, Mni036 developed by Lucas et al. (2009) (see Table 1). DNA was extracted using the ammonium acetate method for adults and larger brood (see Lucas et al. 2011a, b) and the “HotSHOT” method for eggs and small larvae (Truett et al. 2000). Loci were amplified using multiplex PCR, carried out in 6ul reactions using Qiagen PCR master mix (Qiagen Inc.). PCR products were diluted and added to formamide before samples were sequenced using a MiSeq Benchtop Sequencer (Illumina) at the University of Exeter, Penryn. Allele sizes were scored using the software Geneious 10.0.9 (<https://www.geneious.com>). To calculate genotyping error

Table 1 Marker data for the 10 microsatellite loci analysed, k = number of alleles detected in the 488 individuals genotyped, H_E = proportion heterozygotes expected

Locus	Multiplex	k	H_E	Allelic dropout	False allele
Mni002	A	15	0.73	0.069	0.010
Mni003	A	8	0.68	0.024	0.012
Mni009	B	10	0.75	0.050	0.016
Mni017	B	14	0.78	0.038	0.012
Mni019	A	9	0.75	0.043	0.047
Mni024	A	5	0.66	0.021	0.007
Mni028	B	15	0.83	0.000	0.000
Mni030	A	4	0.59	0.036	0.025
Mni034	B	4	0.49	0.060	0.000
Mni036	B	3	0.58	0.048	0.005



Fig. 2 Photographs showing different developmental stages of *Microstigmus* sp. brood; **a** egg; **b** larva; **c** pre-pupa; **d** pupa with light brown eye coloration; **e** pupa with dark brown eye colouration and **f** pupa with black eye colouration

rates, PCRs were repeated for 20% of DNA samples and genotypes compared using the software PENDANT 1.0 (Johnson and Haydon 2007). This software compares duplicate genotypes and uses a maximum likelihood approach to estimate the allelic dropout and false allele error rates based on the nature and number of mismatches identified. False allele rate refers to mis-genotyping due to several factors including human error.

Sex determination

Owing to the haplodiploid sex determination system in Hymenoptera, whereby fertilized eggs develop into female offspring and unfertilized eggs develop into males, brood can be sexed by observing the number of alleles present at microsatellite loci. Thus, brood that appear homozygous at every locus were considered to be haploid males, whereas brood heterozygous at any of the amplified loci were considered to be female. Based on the observed allele frequencies, the probability of a female being homozygous at every locus and thus wrongly assigned as a male was calculated as 3.3×10^{-6} (Boomsma and Ratnieks 1996). Adults can easily be sexed using morphological characteristics (see Field 2023).

Genetic relationships

Using the software RELATEDNESS 5.8.0 (Queller and Goodnight 1989), within nest pair-wise relatedness coefficients were calculated between: all nestmate adult females, adult females to nestmate adult males, adult females to female brood, adult females to male brood and between female brood. Nests were weighted equally, and data were jack-knifed over loci to obtain standard error and 95% confidence intervals.

The statistical estimation of pedigree relationships within nests was performed using the software COLONY which uses a full-pedigree likelihood approach to simultaneously infer parentage and sibling relationships. Three replicate runs were conducted for each population (Santa Lucia or Mashpi) using the following parameters: medium run length, full likelihood, specifying haplodiploid sex determination with female monogamy, male polygamy and error rates calculated from repeat genotyping analysis. To assess the reliability of the results, a different random number seed was used for each replicate run. Relationship assignments were accepted if the same pedigree configuration between two individuals was recovered with an assigned probability of over 90% in at least 2/3 runs.

For female brood, where COLONY assigned either a full-sibling or a mother–offspring relationship to a pair of individuals inconsistently on different runs, an alternative relationship assignment method was implemented using the

software KINGROUP v.2 (Konovalov et al. 2004). We examined whether inconsistently assigned pairs were significantly more likely to be (a) full sisters over mother–offspring, (b) mother–offspring over full sisters, (c) mother–offspring over aunt–niece or (d) aunt–niece over mother–daughter. Full sister relationships were assigned wherever such a relationship was found to be significantly more likely than a mother–offspring relationship, without the opposite being true. Those pairs for which a mother–offspring relationship was significantly more likely than a full-sister (without the opposite being true), were also tested against the likelihood of a maternal aunt–niece relationship. Thus, a pair was assigned as mother–offspring only if this relationship was found to be significantly more likely than both a full-sister and an aunt–niece relationship, without the opposite being true. Those female offspring that could not be allocated to any of the existent adult females were grouped into sibships using COLONY, along with any nestmate adults also found to belong to the full-sibling group. Unresolved female relationships were determined using KINGROUP as described.

As KINGROUP is not able to accurately determine relationships for haploid individuals, males were assigned to sibships only if they could be accurately and consistently assigned as such using COLONY. Male brood that could not be assigned to a mother using COLONY were assigned manually. Relationships between adult females and male brood were assigned as mother–offspring if the male shared an allele at every locus with the focal female. More distant relationships between adult females and male brood were not assigned.

Reproductive skew

In keeping with analyses conducted by Lucas et al. (2011a), we use the index Q presented by Ruzzante et al. (1996) to describe reproductive skew. Q characterizes the observed variance in reproductive distribution as the fraction of the maximum possible variation, with 1.0 representing the maximum variation (i.e., one female being the mother of all brood) and 0 indicating that the brood are equally distributed among females.

Mating frequency

Mating frequency statistics were calculated for the 35 adult females which, using the aforementioned methodology, were found to be the mother of at least two female offspring. We used the software MATESOFT (Moilanen et al. 2004), which calculates the observed and estimated mating frequencies of adult females using the procedures developed by Pedersen and Boomsma (1999). Confidence intervals were obtained by bootstrapping over groups.

Statistical analyses

All statistical analyses were performed using R Version 3.5.3 (<http://www.r-project.org>) and RStudio Version 1.1.463 (<https://www.rstudio.com>). Linear models were performed using the package ‘glmmTMB’ (version 0.2.3; Brooks et al. 2017; R Core Team 2019) which allows for the implementation of generalized linear mixed effect models with various extensions including zero-inflation. Error structures were chosen by fitting all suitable alternatives, given the distribution of the dependent variable in question, and selecting the model giving the smallest AIC.

Results

A total of 651 nests identified as belonging to *Microstigma rosae* were located over an altitudinal range of 771–1950 masl. Most of these nests (81.6%) were found hanging from the underside of *Xanthosoma sagittifolium* plants, 9.5% were located on other unidentified plant species and 8.9% on manmade structures. Despite *X. sagittifolium* being extremely abundant, it was common to find multiple active nests on the same plant, often sharing a single leaf.

Nest architecture

Nests of this species resemble small (max 15 mm in diameter) bag-like structures, approximately teardrop in shape and suspended by a thin, straight, petiole (Fig. 3). Nests are light brown in colour with a rough external appearance, apparently constructed from small pieces of plant materials (such as wood fragments) interwoven with strands of silk. The entrance of the nest is located at the apex, where the petiole meets the main nest structure, and the lower part is divided between 1 and 9 vertically orientated cells (Fig. 3b, $\bar{x} = 3.4$, $n = 222$, $sd = 1.53$) which can be seen from the exterior as bumps on the surface of the nest.

Nests were always founded by a single adult female and appear to be expanded in size according to requirement. This is evidenced through the examination of newly founded nests, where the number of cells is seen to increase progressively over time—presumably in response to accumulating numbers of immature offspring (GLMM, Family: truncated compois, $n = 124$, $LRT_1 = 85.97$, $p < 0.001$).

Brood provisioning and development

Observed brood sizes were small, averaging just 2.44 (\pm SD1.43) offspring per nest (pre-existing nests: range 1–8, $n = 99$). Adult females were found to mass provision their

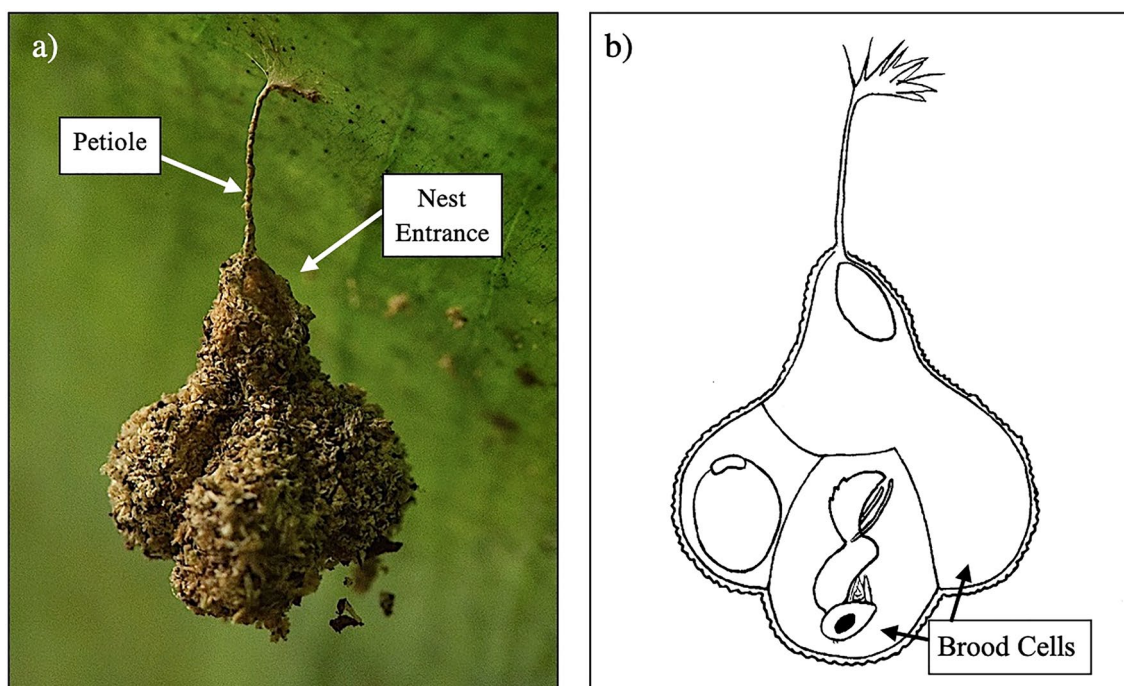


Fig. 3 **a** A *Microstigma* sp. nest hanging from the underside of a *X. sagittifolium* leaf. For scale note that the nest is ca. 1 cm in diameter. Photo Credit: Nick Hawkins; **b** diagram representing the inside of a *Microstigma* sp. nest

offspring with between 31 and 84 individual trips nymphs (order Thysanoptera) which are formed into a compact prey ball upon which a single egg is laid. Completed prey ball—egg assemblages are then sealed into their cell with the same materials used in nest construction. Examination of the rate of offspring accumulation in newly founded nests are consistent with sequential brood production and a foraging period of approximately 10 days between each egg-laying event (Fig. 4a). That said, eight (27%) of the 30 collected multi-female nests were found to contain two offspring of the same developmental stage, thereby indicating simultaneous brood production.

Average nest development was found to be as follows: the nest-building phase lasts around 3 days before provisioning starts, then around 7 days of provisioning takes place before the first egg is laid on around day 10. The egg phase lasts 1–2 days and is followed by a period of around 11 days of larval feeding and growth before offspring enter the prepupal stage. Another 7–8 days pass before the earliest pupal stage is observed. Pupae start to develop brown eye colouration after roughly 3 days, followed by black eye colouration around 2–3 days later (Fig. 4b). The final pupal stage, termed

pre-adult, was not observed in any of the newly founded nests collected. For further details of nest development data and calculations, see Tables S1 and S2.

Offspring sex ratio was significantly female biased ($\chi^2_1 = 14.31$, $p < 0.001$); Of the 161 brood found in pre-existing nests, 105 were female and 56 were male, equating to an observed sex ratio of 0.35 M:F. Adjusting these calculations to incorporate sexual size dimorphism results in an overall investment sex ratio for pre-existing nests of 0.34 M:F (females are on average 5% larger than males, R. Bonifacii & J. Field, unpublished data).

Group size and genetic relatedness

Group sizes were small. The maximum number of females found co-habiting a single nest was four, the maximum number of males was three and the maximum number of adult wasps was six (four females and two males). Of the 163 occupied, pre-existing nests collected, 34 (21%) were found to contain multiple females. There is a highly significant correlation between the number of females and the total number of brood in the nest (Fig. 5a: GLMM, Family: generalised

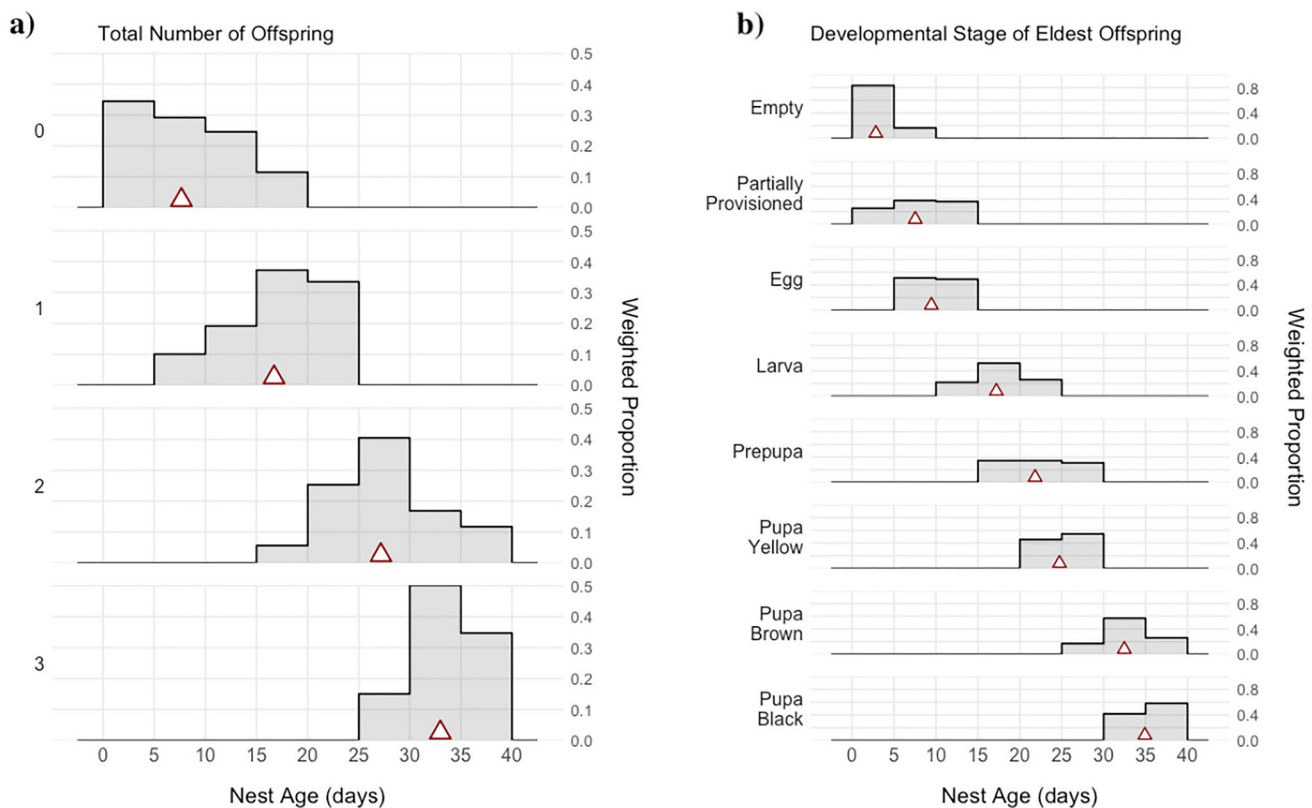


Fig. 4 Histograms showing **a** offspring accumulation and **b** the developmental stages of the eldest offspring within nests, in relation to nest age upon collection. As data are weighted to account for differences in sampling effort, bin height represents the weighted proportion of

observations that fall into each age group. Red triangles indicate the mean number of days from nest founding it takes to reach each developmental stage. Data are truncated at 40 days

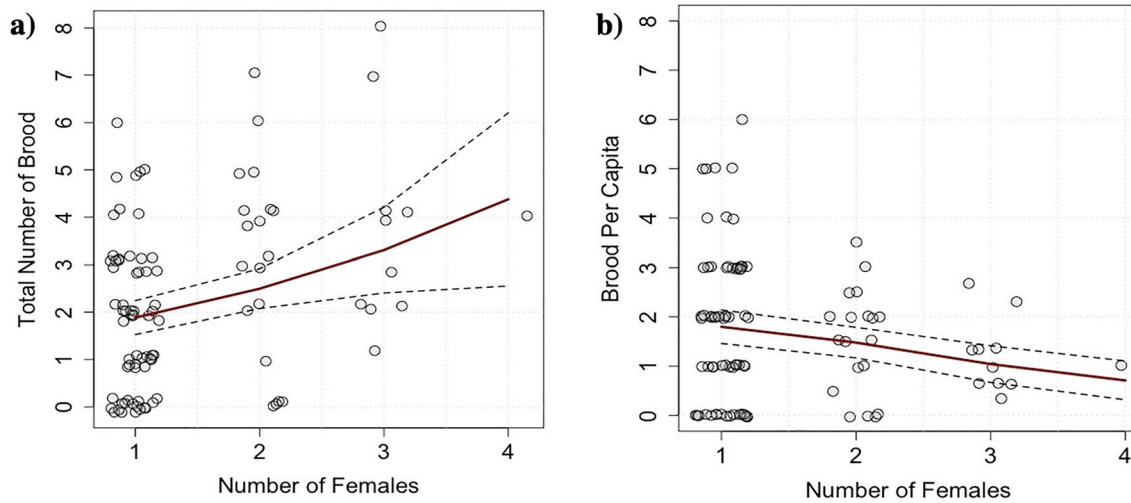


Fig. 5 The number of adult females within a nest is significantly related to **a** the total brood and **b** per capita brood (number of brood per adult female), $n = 99$. Points are jittered for clarity. Red lines indi-

cate the fit of the generalized Poisson GLMM models and dashed lines indicate standard errors

Table 2 Summary of within-nest regression relatedness values by population

Relationship	Mashpi				Santa Lucia			
	<i>n</i>	<i>r</i>	SE	CI 95%	<i>n</i>	<i>r</i>	SE	CI 95%
Adult females	17	0.44	0.04	0.08	22	0.60	0.04	0.08
Adult females to adult males	22	0.50	0.06	0.13	16	0.42	0.08	0.18
Adult females to female brood	96	0.60	0.02	0.04	43	0.56	0.04	0.08
Adult females to male brood	70	0.90	0.02	0.04	33	0.82	0.04	0.09
Female brood	76	0.72	0.03	0.06	44	0.64	0.04	0.08

n number of pair-wise relationships examined; *r* mean Queller goodnight relationship coefficient as calculated by RELATEDNESS; SE standard error obtained by jack-knifing over loci; CI 95% 95% confidence interval obtained by jack-knifing over loci

Poisson, $LRT1 = 11.158, p < 0.001, n = 99$). However, per capita brood (number of brood per adult female) was found to decrease with increasing group size (Fig. 5b: GLMM, Family: generalised Poisson, $LRT1 = 12.24, p < 0.001, n = 99$).

The results of the relatedness analysis are presented in Table 2. Relatedness values among adult nestmate females were high, averaging 0.44 and 0.60 at Mashpi and Santa Lucia respectively, with pairwise values ranging from -0.08 to 1.0 (Fig. 6). Of the 39 pairwise relationships between nestmate females examined, 13 parent-offspring relationships and 17 full-sibling relationships could be confidently determined. The relationships among a further four closely related pairs remain undetermined and may represent either mother-daughters or full siblings. An additional five adult female pairs were found to represent more distantly related individuals.

Relatedness between adult females and female brood was also high at both sites. Obtained values were slightly higher

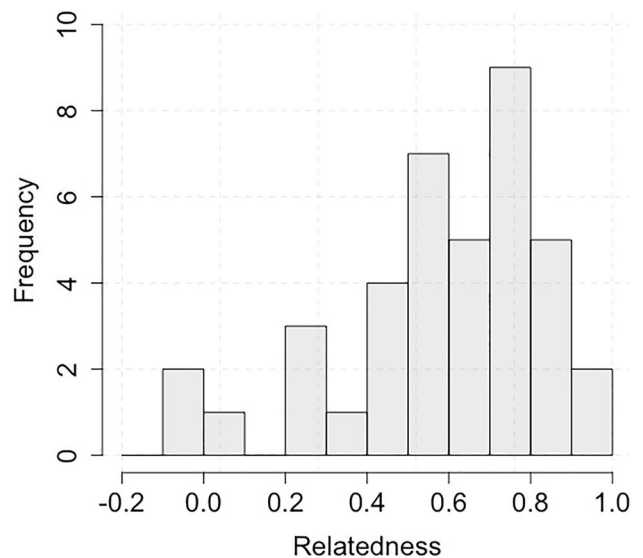


Fig. 6 Histogram of pair-wise relatedness values between nestmate adult females, $n = 39$

than expected for a mother–daughter relationship, likely resulting from delayed dispersal of adult sisters on some nests. Correspondingly, relatedness values between adult females and male brood were slightly lower than expected for mother–son relationships. Relatedness between female brood was close to the value expected for full sisters at Mashpi, and slightly lower at Santa Lucia.

Reproductive skew

For the 23 multifemale nests collected, only 56% (36/64) of offspring could be confidently assigned to an adult female, with an additional three brood for which sister or mother–offspring relationship could not be confidently distinguished. The remaining 24 brood were considered to be orphaned as none of the existing females could be assigned maternity. Twelve of these orphaned brood were found to be the siblings of at least one of the adult females present, six were male brood which could not be assigned to a sibship with COLONY and a further six female offspring were not the siblings or offspring of any of the existing adult females. Overall, only 17 of the 52 females inhabiting multifemale nests were found to have any immature offspring within the nest.

As estimates cannot be made for nests with less than two brood, eight multifemale nests were excluded from our skew calculations. Reproductive skew was found to be high, with a mean Q value of 0.707. 60% (9/15) of nests displayed complete reproductive skew, with a single adult female exclusively responsible for producing all the brood. In the remaining 40% (6/15), reproduction was shared by up to two adult females. In two of these six nests, reproduction was split between one present and one absent female and in a further three, the non-dominant reproductive was responsible for producing only one male offspring. Thus, only one of the fifteen nests provided evidence of two mated females simultaneously contributing to the brood.

Males and mating behaviour

Of the ten nests with adult males where both maternal and paternal genotypes of immature brood could be determined, two of the resident males had genotypes that matched the inferred mate of the egg-laying female. A further five males were likely offspring of the resident female, and three were unrelated.

Multiple mating was found to be uncommon, with only five of the 35 broods analyzed displaying genotypes indicative of multiple mating. All of these had just two inferred patriline. The observed proportion of multiply mated mothers (\pm CI95) as calculated by MATEISOFT was 0.07 (\pm 0.06), the estimated proportion was 0.08 (\pm 0.07) and the average pedigree effective mate number was 1.04 (\pm 0.04). Paternity

skew in nests with multiply mated females (calculated as the proportion of the offspring sired by the mate with the largest contribution to the brood) was 0.6. It is worth noting that because MATEISOFT does not consider genotyping errors when inferring the number of patriline, it is likely to erroneously infer multiple mating wherever such inaccuracies arise. Indeed, closer examination of the inferred paternal genotypes from offspring of apparently multiply mated females revealed that in all five cases, mates differed from each other at only one or two loci. Such observations may indicate that some of the ‘multiply mated’ females reflect genotyping errors, and that multiple mating is less frequent still than these analyses indicate.

Parasitism

Twenty nests (5.7%) were found to contain parasites upon dissection. The number of parasites in a single nest ranged from one to three and all stages of parasite development were observed, from very small larvae to pupae. Parasite larvae were found feeding on *Microstigmus rosae* larvae and pre-pupae but never on pupae. Examination of parasite pupae revealed two distinct forms, one with very long antennae, likely to be braconid wasps of the genus *Heterospilus* (Family Ichneumonidae) (Marsh and Melo 1999) and another with very short antennae, likely a chalcidid wasp (Family Chalcididae). All but one of the parasitized nests belonged to solitary females. However, due to the low frequency of parasitism observed, this represents only a marginally significant trend ($LRT_1 = 2.92$, $p = 0.09$, GLMM, Family: Poisson, $n = 175$).

Discussion

Sociality in *Microstigmus* is thought to represent an independent origin of social behaviour with the potential to provide important contributions to comparative studies of social evolution (Ross and Matthews 1989). Despite this, *Microstigmus* remains understudied and data concerning the biology of sociality are lacking. This paper provides a thorough description of the life history and nesting biology of a new species belonging to this genus, *Microstigmus rosae*. Our findings suggest that *M. rosae* is an early-stage social species; while not uncommon, social groups are small and comprise totipotent individuals which may also live solitarily. Of the three *Microstigmus* species for which we now have in depth data, the expression of social behaviour in *M. rosae* appears to be the most rudimentary. Social group formation is less common, with only 20% of nests containing more than one adult female, compared with around 50% in *M. comes* and 64% in *M. nigrophthalmus* (Matthews 1968; Lucas et al. 2011b). Moreover, reported group sizes

are smaller, with at most four females comprising any one social group, compared to a maximum of 13 and 6 for *M. comes* and *M. nigrophthalmus* respectively (Matthews 1968; Lucas et al. 2011b).

Such early-stage social behaviour may render *M. rosae* especially valuable for the study of social evolution. Studying evolutionary transitions in sociality requires investigations of a broad range of different social stages, from the simplest origins of cooperative breeding through to the complicated elaboration of eusocial societies (Dew et al. 2016). While complex eusocial species have greatly informed our understanding of the processes maintaining sociality, knowledge of the factors associated with its origins may be better investigated through the study of species exhibiting primitive eusociality and other intermediate social phenotypes (Elgar 2015). Species exhibiting social polymorphism are of particular value in this regard as they offer a unique opportunity to explore the factors underlying variation in social organisation. As *M. rosae* is found to inhabit a large altitudinal gradient, this species additionally offers a rare opportunity to explore the impact of ecological conditions on the expression of sociality in this incipiently social species.

Genetic relatedness

Our data suggest that *M. rosae* nests are founded by a single female and that larger group sizes arise when emerging female offspring remaining on the natal nest. Consequently, the majority of nestmate female pairs have either sister or mother–daughter relationships and average within nest relatedness values were high at both sites. These high mean relatedness values are likely to be maintained by the high rates of female monandry observed. Under monandry, brood within nests will be composed of full sibling groups and kinship will be high. By increasing genetic relatedness between family members, monoandry is thought to facilitate the evolution of cooperative behaviour by enhancing the associated inclusive fitness payoffs (Bourke 2014). As a result, such associations between a singly mated female and her female offspring are thought to represent the ideal genetic circumstances for the emergence of social behaviour (Boomsma 2009; Cornwallis et al. 2010; Lukas and Clutton-Brock 2012; Smith et al. 2018).

Male behaviour

Our data suggest that at least some of the time, mating may occur within nests. By taking up residence within nests, it may be that males are better able to find a mate either through mating with the adult foundress or waiting for emerging female offspring. Moreover, these data indicate that prior to dispersing, newly enclosing male offspring remain for a time on their natal nest. Such behaviour may,

therefore, be indicative of a ‘partial localised mating’ life history whereby male offspring mate with emerging sisters before dispersing to find additional mates in the population at large. Although evidence for such behaviour is currently limited, the female-based population brood sex ratio observed for *M. rosae* are consistent with these suggestions. Further genetic investigation into the likely occurrence of sib-mating in *M. rosae* is needed to elucidate the potential role of inbreeding in generating selection for group living in this species.

Costs and benefits of social nesting

That said, indirect fitness benefits alone are unlikely to be sufficient to select for social rather than solitary nesting in *M. rosae*. Although additional females appear to positively impact colony productivity, per capita productivity decreases with increasing group size (Fig. 5). Under these circumstances, selection for social behaviour will also depend on the direct benefits of group living relative to independent reproduction; that is, the underlying factors that determine the ‘*b*’ and ‘*c*’ terms in Hamilton’s rule.

Indeed, genetic analysis has revealed that opportunities for direct reproduction may be an important source of fitness benefits for subordinate *M. rosae* females. Although reproductive skew was found to be generally high within nests, it was not uncommon to find brood that had been produced by multiple females. In most of these cases, however, reproduction was found to either be divided between one active female and one ‘missing’ female, or the non-dominant reproductive was responsible for producing only one male offspring. Thus, it appears that opportunities for direct reproduction may most commonly arise after the current reproductive dominant has left or died. High numbers of orphaned brood, together with the prevalence of nestmate sister pairs, also suggest that nest inheritance is a common occurrence. As nests of *Microstigmus* are constructed using large quantities of silk, a substance that is presumably costly to produce, it seems likely that these structures represent a sufficiently valuable resource that the chance of inheritance may warrant temporary sacrifices to direct reproduction (Piper et al. 1995; Kokko et al. 2001; Shreeves and Field 2002; Leadbeater et al. 2011).

Parental care and provisioning

Unusually among apoid wasps, *Microstigmus* females are nest loyal, ovipositing all their brood in a single nest and remaining at the nest to provide for subsequent offspring. Such behaviour exemplifies another important preadaptation for social behaviour: the occurrence of well-developed parental care. In this context, particular emphasis is placed on the method of brood provisioning, which is thought to

have a significant impact on the necessary duration of such care. Traditionally, progressive provisioning, whereby adults feed offspring gradually throughout their larval period, is thought to extend the duration of parental care and may generate insurance-based advantages to group living (Queller 1994; Field 2005). In contrast, mass-provisioning species, such as *M. rosae*, provide offspring with all the food they need to reach maturity at the beginning of their development. Thereafter, cells are sealed, and it is typically assumed that no further direct interactions with offspring take place. While *M. nigrophthalmus* provisions offspring progressively, the most well-developed example of sociality within *Microstigma*, *M. comes*, is also known to mass provision its offspring. These observations draw into question whether an ancestral state of progressive provisioning is necessary to promote the emergence of social behaviour in this genus.

Food provisioning is not the only form in which parental care occurs. Parental care, broadly defined as “all parental traits that enhance offspring fitness” (Royle et al. 2012), includes other important behaviours such as the creation and maintenance of nests, the protection of immature offspring from predators (Kukuk et al. 1998; Smith et al. 2003) and parasites (Field and Brace 2004), and hygienic brood maintenance. The presence of adults on the nest has been demonstrated to significantly decrease the incidence of brood mortality in several species of mass provisioning sweat bees (Eickwort et al. 1996; Zobel and Paxton 2007), with protection from ant predators demonstrated to be an important component of parental care (Kukuk et al. 1998; Smith et al. 2003). In *M. rosae*, all but one of the 20 occurrences of interspecific parasitism were found to befall single female nests. Although the low frequency of parasitism means that this is only a marginally significant trend, it seems highly plausible that groups of multiple females would provide better protection for developing brood compared to lone individuals that must leave the nest undefended while foraging.

Moreover, although prey ball—egg assemblages are sealed into their cells upon completion, it may be incorrect to assume that this behaviour impedes hygienic brood care by adults. Many species of mass-provisioning halictine bees, for example, are known to re-open cells to remove parasitised, diseased and dead brood (Batra and Bohart 1969; Plateaux-Quénu 2008; Quiñones and Wcislo 2015), with similar behaviour reported in mass-provisioning ceratinine bees (Rehan et al. 2009; Rehan and Richards 2010). Moreover, the distinct lack of faecal matter found inside the cells of developing *M. rosae* larvae suggest that adult females may periodically inspect and clean developing brood. Not only is such hygienic brood maintenance likely to have an impact on offspring survival, thus extending the duration of offspring dependency on parental care, but this behaviour may also reduce the costs of sociality by attenuating the associated increased risk of disease

(Plateaux-Quénu 2008). Hygienic behaviour may, therefore, be an important component of parental care in *Microstigma*, with the potential for significant impact on selection for social behaviour (Socias-Martínez and Kappeler 2019). Together, these observations highlight the need for further investigation into the significance of post-provisioning parental care for offspring survival in this genus.

Conclusion

The results presented in this paper provide a detailed overview of the general biology of *Microstigma rosae*. Through these analyses, we have built a good understanding of social and genetic structure in this species which support several theories concerning the characteristics thought to be important for the emergence of sociality; namely, high relatedness generated by the association of a single mated mother and her female offspring, nest fidelity, hygienic behaviour and the potential for direct reproduction and nest inheritance. In contrast, one hypothesised trait, progressive provisioning, is notably absent, drawing into question whether such behaviour is required to promote the emergence of social behaviour in this genus. The scope of these analyses could be further expanded through the comparative study of the same traits in other *Microstigma* species in a phylogenetic context. However, studying such evolutionary transitions requires detailed knowledge on a wide variety of species, data which are not currently available. Thus, while the documentation of *M. rosae* represents a valuable contribution to this dataset, further understanding of the genetic, behavioural and ecological circumstances favouring sociality in this genus can be achieved only once comprehensive data are obtained for a greater number of species.

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Author contributions Both authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by RB. The first draft of the manuscript was written by RB with comments and editing by JF. JF supervised the project. Both authors have read and approved the final manuscript.

Data Availability Data available under <https://figshare.com/s/aa04d68396318497f02b>.

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