

# Inclusive Fitness of Male and Facultatively Social Female Nesting Behavior in the Socially Polymorphic Bee, *Ceratina australensis* (Hymenoptera: Xylocopinae)

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## Abstract

Male hymenopterans do not typically provide help with nest construction or maintenance. This is thought to be due to the decreased relatedness of males to their siblings compared to sisters, and selection for outbreeding resulting in male dispersal from natal nesting sites. However, some instances of male ‘helping’ behaviors have been observed and can usually be explained by increased access to mating with resident females. Here we report on the first observations of cohabiting males within the nests of reproductive females of the facultatively social small carpenter bee, *Ceratina australensis*. Social nesting in *C. australensis* occurs at a consistently low rate across populations. We used microsatellites markers to determine relatedness, combined with 3 yr of nest demographic data collected across three populations, to assess the relative fitness of reproductive, nonreproductive, and male individuals cohabiting in reproductive nests. We found that males were brothers of reproductive females, both remaining in their natal nest. However, there was no evidence that they were mating with their sisters across all nests observed. Males in reproductive nests did not gain any direct or indirect fitness benefits as they did not sire any brood and their presence did not increase brood productivity or survivorship. It is possible that males were waiting to mate with nieces who had not yet emerged. Why males were tolerated remains unknown. Mating biology is an important consideration in social theory which requires additional empirical studies. Future long-term studies are needed to capture unusual social behaviors including male nesting behaviors.

**Key words:** small carpenter bee, relatedness, male helpers, mating behavior, diploid males

The development of a sterile worker caste represents a significant evolutionary transition for life on earth (Wilson 1975). Costly behaviors such as altruism can be adaptive if the level of inclusive fitness gained by altruists outweighs the costs of forgoing direct reproduction. Over evolutionary time and given adequate environmental circumstances, incremental fitness gains by altruists and related kin can fix altruistic phenotypes within populations (Crozier and Pamilo 1996). Such fixation is evident within highly social forms such as ants (Formicidae) and honey bees (*Apis mellifera*) who have passed the evolutionary ‘point of no return’ (Wilson 1971). Taxa which have not undergone such intense phenotypic fixation however are widespread within the bees. Sociality has evolved and been lost multiple times within the bees (Danforth 2007, Rehan et al. 2012) and is therefore a highly plastic trait dependent on phylogenetic inertia and environmental constraint (Paxton et al. 2002, Cronin and Hirata 2003, Purcell 2011, Cornwallis et al. 2017, Groom and Rehan 2018).

Inbreeding is thought to be an early driver of social evolution by increasing relatedness within groups and the inclusive fitness

benefits of helping by altruists (Hamilton 1972, Trivers and Hare 1976). However, due to the complementary sex determination (CSD) of Hymenoptera, inbreeding can also increase the proportion of sterile diploid males within a population (Cook and Crozier 1995). Males are produced through CSD via hemizyosity at sex-determining loci (Harpur et al. 2013). CSD is thought to be ancestral to the Hymenoptera (Asplen et al. 2009). Diploid males are produced when individuals are homozygous at CSD loci, and such males are potentially infertile and could represent a loss of fitness for reproductive females (Cook 1993, but see Cowan and Stahlhut 2004). Populations with a small effective size are particularly susceptible to increased diploid male production as the effects of drift potentially decrease CSD allelic richness (Cook and Crozier 1995). However, empirical data on sufficient male diploidy affecting the fitness of bottlenecked populations are lacking (Kukuk and May 1990, Elias et al. 2009, López-Urbe et al. 2016).

Highly social hymenopteran (ant, bee, and wasp) societies are structured around related female individuals of multiple generations.

Due to the hymenopteran haplodiploid sex-determination system, daughters of singly mated females share 75% of their genes, while sons share 50%. In line with predictions of inclusive fitness theory (i.e., kin selection; Hamilton 1964, 1972), male hymenopteran helping behavior is rare or infrequent (Mikát et al. 2019). Examples include male fanning and nursing behavior under natural conditions in at least three different species of *Polistes* wasps (Hunt and Noonan 1979, Cameron 1986) and nursing behavior under experimental removal of females in the wasp *Ropalidia marginata* (Sen and Gadagkar 2006). In both *Polistes* and *Ropalidia* it was observed that males masticated food sources for longer, most likely imbibing more of the food, than females feeding larvae (Hunt and Noonan 1979; Cameron 1985, 1986; Sen and Gadagkar 2006). Cameron (1985) has also observed male incubation of pupae in natural and cage nests in two species of *Bombus*.

Direct fitness benefits of helping could exist for hymenopteran males if helping allows for greater mating opportunities. For instance, certain male wasps in the families Crabronidae and Sphecidae patrol and/or passively defend nest sites from both brood parasitism and competing males (Lin and Michener 1972, Peckham 1977, Lucas and Field 2011), thus increasing the direct fitness of males and female wasps. Additionally, the Australian halictid sweat bee *Lasioglossum hemichalceum* contains a male dimorphism thought to be related to nest defense (Houston 1970). However, in the absence of behavioral observations and the frequency of within-nest mating (Kukuk and Sage 1994), it seems more likely that emergence of this male dimorphism is related to sexual preference of females. *Trypoxylon montevedae* wasps provide evidence for hymenopteran paternal care as male–female pairs were observed cooperating in nest maintenance and defense (Brockmann 1992). Certain behaviors, such as males smoothing out of mud walls, were not seen done by females when a male was present (Brockmann 1992) indicating the potential for a rudimentary type of division of labor. However, male–female pairs were long-lived and were observed copulating within the early stages of nest construction. Therefore, when male cooperative behaviors are observed in natural settings, they are likely due to increased access to mating opportunities, as opposed to a behavior resembling altruism (Lin and Michener 1972, Brockmann 1992, Lucas and Field 2011).

The small carpenter bees of the genus *Ceratina* (Apidae: Ceratinini) are a cosmopolitan genus characterized by high social variability (Michener 1990). All studied ceratinines are subsocial, meaning that mothers provide extended care for offspring until they reach reproductive maturity (Sakagami and Maeta 1984, Michener 1990, Maeta et al. 1997, Rehan and Richards 2010, Rehan et al. 2010). Maternal care behaviors, such as foraging and nest construction or maintenance, are considered vital preconditions for the evolution of social behavior (Wade 2001). As such, the ubiquity of subsociality and the variability of sociality within the ceratinines underscore their position as a unique clade for understanding the evolution of parental and social behaviors (Rehan et al. 2014a, Groom and Rehan 2018).

The Australian small carpenter bee, *Ceratina* (*Neoceratina*) *australensis* Perkins, is a facultatively social bee with a bivoltine life cycle (Rehan et al. 2010, 2011, 2014b; Dew et al. 2018); a relatively small percentage of collected nests within a population contain two adult female sisters, one of whom does not reproduce (Rehan et al. 2014b). It is hypothesized that high variability in parasitism rates between years has fixed limited dispersal, the phenomenon in which one sister does not leave her natal nest, in *C. australensis* (Rehan et al. 2014b). In this scenario, the nonreproductive female (the social secondary) could gain indirect fitness benefits by protecting

the brood of her sister (the social primary) from parasite attack, thereby increasing per capita brood production of social nests compared to solitary nests. There is also the possibility that social secondaries can inherit their sisters' nest in the case of the primary's untimely demise and could then presumably care for her sister's brood until they disperse. However, if the primary did not die, this situation would come at a great energetic cost to the primary who forages for and feeds her sister. Previous analyses of *C. australensis* nesting strategies across its range have found that while social nests can have higher brood survivorship (Rehan et al. 2014b), neither per capita brood production nor inclusive fitness benefits to social secondaries explain the persistence of social nesting in this species across years (Rehan et al. 2014b). As the majority of social nests were found in reused nests (Rehan et al. 2010), it is likely that dispersal patterns play a role in the social organization of this species. However, the exact mechanism behind these dispersal patterns remains less clear.

In this study, we report the presence of cohabiting males within nests of *C. australensis*. Given the rarity of male alloparental behavior within the Hymenoptera (Bartz 1982), we predicted that males were fathers to offspring observed in active reproductive nests. If males were not fathering offspring but shared a high degree of relatedness to nestmates, this may indicate a rare case of male altruism. We tested these predictions using eight polymorphic microsatellite loci (Oppenheimer et al. 2018) combined with 3 yr of nest demographic information across the species' range. The presence of males in nests also allowed us to reanalyze the costs and benefits of different nesting strategies given a different set of years and the addition of a newly discovered social arrangement. We approached this analysis through comparing 1) the proportion of each social strategy in reused versus new nests, 2) nest productivity between each strategy, and 3) the degree of relatedness ( $r$ ) between individuals in a nest. We then took this information to calculate the indirect fitness of each strategy an individual bee could take (solitary, social primary or secondary, or cohabiting male).

## Methods

### Nest Collections and Assessments

*Ceratina australensis* were collected from nests formed in the pith of dead stems from three populations across Australia: near Warwick, Queensland (QLD: 28.24°S, 152.09°E); near Mildura, Victoria (VIC: 34.15°S, 142.16°E); near Adelaide, South Australia (SA: 34.94°S, 138.50°E) in January 2015–2017. Sticks were refrigerated to sedate nest inhabitants prior to opening. Nest contents were assessed by splitting sticks lengthwise and recording the cell position and developmental stage of the brood. Nests were then classified as having been recently founded (FN), actively provisioned (AB), or as containing full brood (FB) or mature brood (MB) according to Rehan et al. (2010). FN nests contain a single female and no pollen. AB nests contain cells that are actively being provisioned, while FB nests are identifiable by the presence of a larva or pupa in the youngest brood cell. MB nests contain callow offspring which cohabit with adult bees (Rehan et al. 2010). Bees were killed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

Social status of nests at the AB or FB stage was classified based on adult number and composition. Solitary nests contained only one adult female, while social nests contained two to four females. Bisex nests contained one to three females and never more than one male. Reuse of nesting burrows from a previous brood-rearing period can be determined by darkened nest walls, stained with frass

and/or uneaten pollen; new nests have clean walls without stains. Females in solitary FB nests were conservatively inferred to be secondary females which had inherited social nests, if females had no wing damage (score = 0), and advanced stage brood was present (at least one pupa) explicitly in basal brood cells. As *C. australensis* lay eggs from the bottom of the nest upwards, the presence of pupae toward the front of the nest would leave open the possibility that unworn females were recently emerged offspring, not social secondaries (Rehan et al. 2014b).

Nest productivity was assessed by overall clutch size (CS), the number of live brood (LB), survivorship and per capita brood production (PCBP). Clutch size is the total number of cells containing developing brood within a nest, while live brood is the number of live offspring at the time of collection. Survivorship is equal to LB divided by CS and is the proportion of brood that was not parasitized or dead for any other reason. We divided both clutch size and live brood by the number of adult bees in each nest to calculate two measures of PCBP.

Male *C. australensis* can be identified by examining the morphology of the ventral side of the last abdominal segment (S7 in males, S6 in females), which terminates in the form of two distinct lobes (a 'W' shape), whereas female S6 terminates in a point. Adults, callow offspring, and pupae were all sexed under a light microscope. The body sizes of all adult, callow offspring, and pupae were measured using head width, which is an accepted proxy for overall body size (Rehan et al. 2010). Wing wear, as a measure of nicks and tears in the wing margin, was scored from 0 (no damage) to 5 (very worn), following Rehan et al. (2009, 2010), as a proxy for age and foraging effort (Cartar 1992) for both males and female adults. Female abdomens were dissected in 70% ethanol and ovary development measurements were performed by taking the sum of the lengths of the three largest terminal oocytes. Ovarian development and wing wear status were then used to help classify adult females in social nests as either reproductives or nonreproductive. Social primaries have increased ovarian development and wing wear scores compared to social secondaries (Rehan et al. 2010). Wing wear of males was compared to the wing wear of females and males taken from FN and MB nests to assess the flight effort of cohabiting males compared to males and females from the population at large.

### Genotyping and Relatedness Assessments

In total, 53 nests were genotyped at eight microsatellite loci developed specifically for *C. australensis* (Oppenheimer et al. 2018). This included 29 nests from SA, 14 nests from QLD, and 10 nests from VIC. We genotyped 21 solitary nests, 17 social nests, and 13 nests containing males. Pairwise relatedness coefficient ( $r$ ) values were calculated using KinGroup (Kononov et al. 2004) using the Queller–Goodnight estimation method (Queller and Goodnight 1989) and empirical allele frequencies across all populations. The Queller–Goodnight method produces relatedness values which range from  $-1$  to  $+1$ . Negative values indicate that paired individuals share fewer alleles than a randomly drawn pair (Queller and Goodnight 1989). To accommodate the formula, male–female relatedness estimates were calculated by inputting haploid males as diploid. Estimated  $r$  values were then divided in half to compensate for haplodiploid asymmetry (Trivers and Hare 1976).

Individuals with more than four loci that did not amplify were removed from this analysis. Eggs and larvae, which could not be sexed based on morphology, were sexed based on genotype. Because males are haploid, individuals homozygous across all eight loci were considered male. However, out of a total 124 adults and pupae

visually confirmed as female, 13 (10.5%) were homozygous across all eight loci (all of which were from SA). Of the 32 larvae and eggs from SA, 11 were homozygous across all loci. Assuming a 10.5% female homozygosity rate, only around one of these 11 individuals is likely to be female. As such, we felt confident in classifying all fully homozygous brood as male.

We calculated the expected frequency of diploid males per population as the observed frequency of diploid males per population divided by the probability that an individual is heterozygous for at least one loci ( $P_{het}$ ).  $P_{het}$  can be calculated with the equation:

$$1 - \prod_{j=1}^L \left( \sum_{i=1}^N p_{ij}^2 \right)$$

in which  $p_{ij}$  is the frequency of the  $i$ th allele at the  $j$ th loci,  $N$  is the number of alleles at a locus, and  $L$  is the number of loci (Kukuk and May 1990).

Inspection of genotype profiles also revealed incidences of multiply mated females and the presence of unrelated individuals within nests. We conservatively estimated that a female had mated with more than one male if a putative secondary paternal allele was detected across at least two loci. Unrelated individuals were detected if females consistently differed from a nestmate across at least two loci. Nests collected with developing brood but without an adult were considered orphaned.

### Fitness Calculations

Our fitness calculations were adapted from Rehan et al. (2014b). The fitness of the reproductive female in any given nest type was estimated as the average number of live brood produced per reproductive strategy multiplied by the average relatedness of reproductive females to their brood, multiplied by the probability of her survival. The probability of female survival was calculated as  $1 -$  the probability of nest abandonment (see Results) for solitary females and  $1 -$  probability of nest inheritance for nests containing at least two females.

$$Reproductive = LB * r(LB) * P(survival)$$

Indirect fitness benefits for each social secondary was calculated as their relatedness to brood, multiplied by the probability that the primary female survived, multiplied by the additional live brood produced in social compared to solitary nests ( $b$ ). If social secondaries in social and bisex nests inherited an abandoned nest, they stood to gain direct fitness benefits. Direct fitness of social secondaries in social and bisex nests was equal to the difference in live brood between inherited and social nests ( $b.inherit$ ), multiplied by the expected secondary female's relatedness to her own offspring ( $r = 0.50$ ) multiplied by the probability that the primary female would not survive ( $P(NI)$ ) divided by the number of secondary females within a nest ( $n$  sec).

$$Secondary = b * r(LB) * P(survival) + b.inherit * r(offspring) * \frac{P(NI)}{n \text{ sec}}$$

Indirect fitness benefits for males was calculated in the same way as for social secondaries, substituting the additional number of live brood produced in bisex nests compared to solitary nests ( $b.male$ ) for  $b$ . Males cannot provision their own nest, so they do not stand to benefit from female mortality. However, they do stand to gain direct fitness if they are the offspring's father, in which case direct fitness is equal to the male's relatedness to his offspring multiplied the number of offspring sired.

$$Male = b.male * r(LB) * P(survival) + r(offspring) * n \text{ offspring}$$

## Statistical Analyses

Statistical tests were carried out in R v1.1.383 (R Core Team 2017). To control for the possibility that adults in social and bisex nests were first-brood offspring that would disperse before the end of the FB stage, we compared the proportion of each nesting strategy that was collected in the FB stage to those collected in the AB stage. To test the hypothesis that limited dispersal of first-brood offspring leads to social nest formation (Rehan et al. 2010), we compared the proportion of each nesting strategy that was collected in reused nests to those collected in new nests; chi-square tests were used to compare the proportion of nesting strategies detected between brood development stages (AB and FB) and nest reuse patterns (new vs reused). Significant chi-squares were analyzed using post hoc comparisons implementing Bonferroni correction for multiple comparisons. Clutch size, number of live brood, survivorship, per capita brood production (PCBP), and wing wear scores were compared across reproductive strategies using nonparametric Kruskal–Wallis chi-squares followed by pairwise Wilcoxon signed rank tests. The relatedness estimates for specific pairwise relationships were compared using ANOVAs and Welch's two-tailed *t*-tests.

## Results

In total, 581 AB/FB nests were collected, including 525 solitary nests, 39 social nests, and 17 bisex nests. Table 1 shows the percentage of AB/FB nests collected for each site and year that were social and bisex. We genotyped 37 morphologically identified males in total from across the *C. australensis* range (QLD: *n* = 7; VIC: *n* = 4; SA: *n* = 26). Two adult males from SA collected from two separate 2016 bisex nests were diploid at one locus; no other diploid males were detected in our screen. Therefore, the frequency of diploid males detected in SA was 7.7%. The probability that an individual is heterozygous in at least one loci ( $P_{\text{het}}$ ) for SA was 0.892 while for QLD  $P_{\text{het}} = 0.998$  and for VIC  $P_{\text{het}} = 0.995$ . The expected frequency of diploid males for SA equals 8.6%.

The proportion of nests in the FB compared to AB stage did not differ by nesting strategy ( $X^2_2 = 1.16$ ,  $P = 0.56$ ). The proportion of reused nests varied significantly by reproductive strategy ( $X^2_2 = 23.2$ ,  $P < 0.0001$ ). Nine out of 14 bisex (64%) and 22 out of 34 (65%) social nests were found in reused nests and these proportions did not differ from each other ( $X^2$  post hoc  $P = 1.0$ ). By comparison, only 132 of 482 (27%) solitary nests were found in reused stems; a significantly reduced proportion compared to bisex ( $X^2$  post hoc  $P < 0.05$ ) and social nests ( $X^2$  post hoc,  $P = 0.0005$ ).

## Caste Assessment and Brood Production

Wing wear (WW) did not differ between AB and FB males (AB WW =  $0.375 \pm 0.18$ , *n* = 8; FB WW =  $0 \pm 0$ , *n* = 5;  $t = 2.1$ , *df* = 7,  $P = 0.08$ ). Wing wear differed significantly among sexes and social roles (Kruskal–Wallis  $X^2_2 = 30.9$ ,  $P < 0.0001$ ). Social primaries had significantly greater WW (*n* = 10,  $3.9 \pm 0.53$ ) compared to social secondaries (*n* = 10,  $1.4 \pm 0.57$ ; Wilcoxon,  $P = 0.013$ ), solitary females (*n* = 24,  $1.5 \pm 0.3$ ; Wilcoxon,  $P = 0.002$ ), and males from bisex nests (*n* = 13,  $0.25 \pm 0.12$ , Wilcoxon,  $P < 0.0001$ ). Males from bisex nests had significantly less WW than FN/MB males (*n* = 159,  $1.8 \pm 0.15$ ; Wilcoxon,  $P = 0.005$ ), FN/MB females (*n* = 367,  $1.4 \pm 0.9$ ; Wilcoxon,  $P = 0.03$ ), and solitary females (Wilcoxon,  $P = 0.005$ ), as well as less WW compared to primary bisex females (*n* = 12,  $2.3 \pm 0.70$ ; Wilcoxon,  $P = 0.08$ ) and social secondaries (Wilcoxon,  $P = 0.08$ ), but these differences were not significant. WW between secondary bisex females (*n* = 8,  $0.13 \pm 0.13$ ) and bisex males did not differ (Wilcoxon,  $P = 0.54$ ).

Three percent (6/198) of reused FB nests containing one female were inferred to have been inherited by social secondary females. In total, 6% (35/581) of all solitary AB and FB nests were considered orphaned as no female was present upon collection. There was a significant difference in the live brood size between AB nests (*n* = 360,  $2.14 \pm 0.11$ ) compared to FB nests (*n* = 209,  $2.91 \pm 0.14$ ; Welch's  $t = -4.31$ , *df* = 443.96,  $P < 0.0001$ ). Thus, for all nest productivity and fitness calculations, values were taken from FB nests.

The clutch sizes and live brood numbers produced by solitary females (*n* = 186), primary females in bisex nests (*n* = 6), social primaries (*n* = 11), and secondary inheritors of social nests (*n* = 6) did not differ significantly (Fig. 1; CS Kruskal–Wallis  $X^2_3 = 3.05$ ,  $P = 0.38$ ; LB Kruskal–Wallis  $X^2_3 = 1.64$ ,  $P = 0.65$ ). However, survivorship was greater in solitary nests (mean  $\pm$  SE =  $0.91 \pm 0.02$ ) compared to bisex nests ( $0.70 \pm 0.10$ ; Fig. 1; Kruskal–Wallis  $X^2_3 = 10.03$ ,  $P = 0.02$ ; Wilcoxon solitary–bisex survivorship,  $P = 0.004$ ). All other survivorship comparisons were not significantly different at  $P < 0.05$ . PCBP significantly differed by reproductive strategy (CS PCBP Kruskal–Wallis  $X^2_2 = 9.70$ ,  $P = 0.01$ ; LB PCBP Kruskal–Wallis  $X^2_2 = 14.4$ ,  $P < 0.001$ ). Overall, solitary PCBP was greater than nests containing multiple adults; however, bisex nest clutch size PCBP was not significantly different from solitary clutch size PCBP (Fig. 2; Wilcoxon solitary–bisex clutch size PCBP,  $P = 0.16$ ).

## Relatedness Estimates

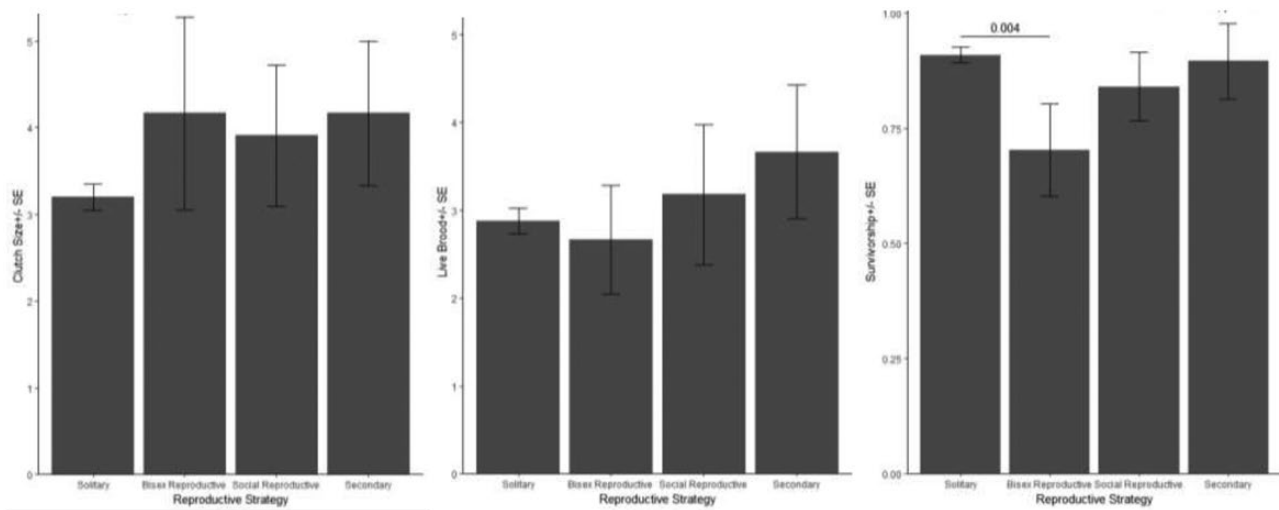
Of the 23 nests containing a single female that were genotyped, two contained a single male offspring that shared low relatedness with ( $r = 0.06$  and  $-0.05$ ) and differed from the resident female by at

**Table 1.** Social nesting composition of AB/FB nests collected across the range of *C. australensis* from 2015 to 2017

Site	Year	Number of AB/FB nests	Number of social nests	Percentage social	Number of bisex nests	Percentage bisex
QLD	2015	55	2	3.6	0	0.0
	2016	86	3	3.5	2	2.3
	2017	50	5	10.0	0	0.0
SA	2015	154	4	2.6	1	0.6
	2016	147	17	11.6	13	8.8
	2017	26	1	3.8	1	3.8
VIC	2015	19	0	0.0	0	0.0
	2016	29	3	10.3	0	0.0
	2017	15	4	26.7	0	0.0
Total		581	39	6.7	17	2.9

The percentages of social and bisex nests are out of the number of AB/FB nests collected within each site and season. Bisex nests were predominately found within SA in 2016.





**Fig. 1.** Reproductive productivity of different nesting strategies observed by *C. australensis*. There was no difference in average clutch size or average live brood by nesting strategy at  $P < 0.05$ . Solitary nests had a significantly greater proportion of offspring alive, not parasitized or visibly dead, at the time of collection compared to bisex nests ( $P = 0.004$ ). No other pairwise comparison was significant. Sample size for each strategy are: solitary 186; bisex 6; primary 11; secondary 6. Error bars represent standard error.

least one locus. These nests were inferred to have been inherited by nonkin individuals. Evidence of two patrilineages was detected in the brood genotype profiles of five of the remaining 21 solitary nests (24%). Multiple mating was not detected in any other nest type. Average estimated relatedness among female offspring (sisters) was significantly greater in singly mated solitary nests ( $r = 0.77$ ) compared to multiply mated solitary nests ( $r = 0.47$ ; Table 2).

Of the 17 social nests genotyped, five contained 3–4 females (there were eight such social nests across the three populations and 3 yr overall—one from QLD 2017, two from VIC 2016/2017, and five from SA 2016). One nest containing four females (QLD 2017) contained two sisters ( $r = 0.69$ ) alongside two unrelated females ( $r = 0.12$ ; 95% CI:  $-0.28$ – $0.52$ ,  $n = 5$ ). Similarly, two nests from SA containing two females did not share alleles at one locus and had low relatedness estimates (0.31 and 0.12), while an additional social pair from QLD in 2015 differed at two loci ( $r = -0.52$ ) suggesting that these three nests contain unrelated social pairs. In total, four social nests contained unrelated adult females. Interestingly, females in nests containing 3–4 females shared significantly higher  $r$  values ( $r = 0.83$ ;  $df = 2, 33$ ,  $F = 10.9$ ,  $P = 0.0002$ ) compared to females in nests containing two females ( $r = 0.48$ ; Tukey HSD =  $-0.35$ ,  $P = 0.0003$ ) but did not differ from social females in bisex nests ( $r = 0.81$ ; Tukey HSD =  $0.02$ ,  $P = 0.95$ ). Social females in bisex nests were significantly more related compared to social pairs (Tukey HSD =  $-0.3$ ,  $P = 0.002$ ; Table 2). Female offspring (sisters) in social nests, regardless of the number of females, were related by 0.74 on average.

Adult females in bisex nests were related to resident males by 0.29. This value (0.29) was not appreciably different from sister–brother pairs from solitary nests ( $r = 0.26$ ) or social nests ( $r = 0.34$ ; Table 2). Males differed from female offspring by at least one locus in four bisex nests that contained female offspring. Male–female offspring were related by 0.19 (95% CI:  $0.07$ – $0.31$ ,  $n = 8$ ). The only lone sister offspring pair collected and genotyped from any bisex nest was related by 0.66.

### Inclusive Fitness

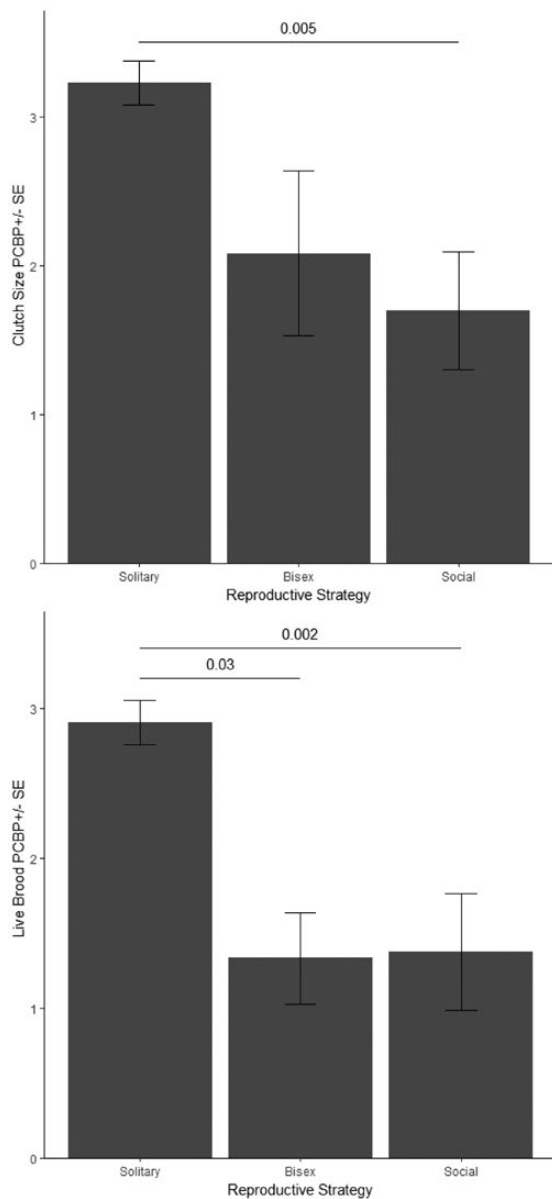
The average number of live brood (LB) collected from AB nests was lower than live brood from FB nests indicating that a reproductive mother's survival to the end of the reproductive period should

impact her fitness. Fitness calculations were thus taken only from FB nests. The probability of solitary nest abandonment was taken as the product of the proportion of orphaned nests (35/581 solitary nests or 6.0%) and the proportion of nests containing alien female genotypes (2/23 genotyped solitary nests or 8.7%). The probability of female survival was thus  $1 - \text{probability of nest abandonment}$  (0.005) for solitary females (0.995). The probability that social secondaries would not inherit the nest was  $0.97$  ( $1 - \text{probability of nest inheritance}$  or  $1 - 0.03$  (6/198 reused nests)). The fitness of solitary females was  $1.34 \pm 0.07$ , social primaries was  $1.54 \pm 0.39$ , and bisex primaries was  $1.25 \pm 0.28$ .

As there was no additional live brood produced in social or bisex nests, the indirect fitness of social secondaries was equal to zero. The potential benefit of inheriting an abandoned nest was equal to  $b$ .  $\text{inherit} = 3.67 \text{ LB per inherited nest} - 2.87 \text{ LB per social nest} = 0.8$ . The fitness of social secondaries was  $0.05 \pm 0.03$ .

Relatedness estimates for males and females suggest that males are brothers who have not dispersed (Table 2) and could therefore benefit from indirect fitness if they are helping to support more live offspring than females could produce without them. The low wing wear scores of males further suggest that they are not often departing the nest which, for males, would likely imply mate-searching flights. Males appear to be playing a similar behavioral role to nonreproductive females (i.e., potential nest guard) and their presence did not increase the number of live brood (Fig. 2). They would therefore receive no indirect fitness. There was no direct evidence that males were mating with resident females. If males were waiting for the emergence of their nieces for mating opportunities ( $n$  offspring), the direct benefits of this strategy would not have been evident to us at the time of nest collection. Male inclusive fitness is therefore equal to zero.

The fitness of social secondaries was significantly lower compared to reproductive females (Kruskal–Wallis  $X^2 = 37.9$ ,  $P < 0.0001$ ). Social secondary fitness ( $n = 13$ ) was significantly lower compared to solitary fitness ( $n = 186$ ;  $W = 2,405$ ,  $P < 0.0001$ ), social primary fitness ( $n = 11$ ;  $W = 143$ ,  $P < 0.0001$ ), and bisex reproductive fitness ( $n = 6$ ;  $W = 78$ ,  $P < 0.001$ ). The fitness of reproductive females between nest types was not significantly different (bisex–social:  $W = 28$ ,  $P = 0.6$ ; bisex–solitary:  $W = 462$ ,  $P = 0.5$ ; social–solitary:  $Z = 864$ ,  $P = 0.38$ ).



**Fig. 2.** When controlling for the number of adults within each nest, PCBP differed between nesting strategies. Bisex nests produced an intermediate number of offspring per adult compared to solitary, social, and multi-female nests when using clutch size but had significantly fewer number of live brood produced per individual. Numbers above line segments connecting strategies are *P*-values for significant pairwise Wilcoxon signed rank tests.

## Discussion

We analyzed the presence of cohabitating males within nests of a facultatively social bee using microsatellite markers. Adult males and females were highly related yet there was no evidence for inbreeding within these nests. Inbreeding from pre-hibernal assemblages (MB) has been documented within the genus *Ceratina* once before (*C. flavipes*, Kidokoro et al. 2003), as has male guarding by blocking the nest entrance (*C. strenua*, Kislow 1976; *C. smaragdula*, Hefetz et al. 1979). Only one previous study has assessed the relatedness and male cohabitation within active and full brood nests of this genus (*C. nigrolabiata*, Mikát et al. 2019). This latter study suggests males could gain access to direct

breeding opportunities with relatives or delayed breeding opportunities coupled with indirect fitness benefits through increased brood productivity (Mikát et al. 2019). In this study, we found that brood production did not increase in nests with multiple nesters, and brood survival was lower in nests with males compared to solitary nests (Figs. 1 and 2). Unlike Mikát et al. (2019), we found no evidence for inbreeding or direct fitness of males who also received no inclusive fitness benefits. Nonreproductive females received minimal inclusive fitness benefits and were similar to males as both had less wing wear compared to social reproductives. It is possible that males were waiting to mate with nieces or were delaying mate-searching flights until the end of the second-brood period.

It is peculiar that males were not forcefully removed from nests by females, as their presence does not seem to benefit resident females and may rather decrease the survival rate of live brood (Fig. 1). Males of *Xylocopa* large carpenter bees remain within their nests for the first 2–3 wk of eclosion (Gerling et al. 1981). They are usually forced from entering nests by physical force of females (Minckley and Buchmann 1990), most likely because returning females feed nestmates upon return from foraging trips and males do not contribute to nest defense or upkeep (Michener 1972, Hogendoorn and Velthuis 1993). *Ceratina* females similarly feed adult nestmates (Rehan et al. 2014a, Mikát et al. 2017, Shell and Rehan 2018). Males were probably fed by returning reproductive females, as WW scores suggest minimal foraging flights, decreasing the amount of food remaining for brood production. However, decreased survival was not observed in social nests where adult food transfer would also likely occur indicating that the decreased survival in bisex nests observed in this study is not a sole effect of decreased food availability for offspring.

## Social Nest Formation

Limited dispersal of females from natal nests is hypothesized to lead to social nesting in *C. australensis* (Rehan et al. 2010, 2011, 2014b), and nesting in aggregations can lead to rudimentary forms of social behavior even in the absence of high relatedness or overt fitness benefits (Abrams and Eickwort 1981, Taylor 1992). Consistent with this hypothesis we found that the frequency of nest reuse was higher among social and bisex nesting strategies than observed in solitary nests. While a high proportion of social and bisex nests were found in reused nests, 40% of these nests were found in new nests. This indicates that social nest formation may be dependent on limited dispersal from natal nest aggregations, and not necessarily from natal nests per se. In line with this theory, we also detected a moderate instance of drifting: 11% (6/53) of genotyped nests contained an alien female, including 23.5% (4/17) of social nests. Drifting between nests is common within social Hymenoptera (Schwarz 1987, Yanega 1990, Soro et al. 2009, Ulrich et al. 2009, Leadbeater et al. 2011, Yagi and Hasegawa 2012) and offers potential benefits through competition avoidance with related nestmates or potential inheritance in new nests.

Our genetic data for bisex nests suggest that males resemble brothers to resident females ( $r = 0.29$ ), and that resident females are full sisters ( $r = 0.81$ ). Both seem to be related from a singly mated mother and remaining in their natal nest, consistent with the hypothesis of limited dispersal as a mechanism for social nest formation in *C. australensis*. However, relatedness coefficients between pairs of social females were lower than expected for full sisters of singly mated females (Table 2; Trivers and Hare 1976). We detected

**Table 2.** Relatedness coefficient estimates calculated for relationships across *C. australensis* range

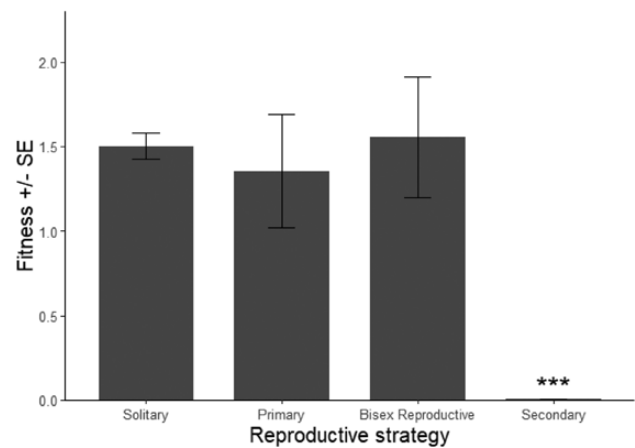
Relationship	Mono solitary	<i>n</i>	<i>n</i> nests	Multi solitary	<i>n</i>	<i>n</i> nests	df	<i>t</i>	<i>P</i>		
Female offspring	0.77 (0.70–0.83)	36	13	0.47 (0.26–0.68)	27	11	31.5	2.7	<b>0.01</b>		
Female–male offspring	0.22 (0.18–0.26)	63	6	0.36 (0.28–0.44)	25	4	38.4	–3.17	0.003		
Relationship	Social	<i>n</i>	<i>n</i> nests	Social	<i>n</i>	<i>n</i> nests	df	<i>t</i>	<i>P</i>		
	2 females			3–4 females							
Female offspring	0.76 (0.67–0.85)	18	2	0.69 (0.58–0.81)	6	1	15.04	1.08	0.3		
Social females	0.48 (0.31–0.66)	9	9	0.83 (0.76–0.92)	15	4	11.76	–4.21	<b>0.001</b>		
Relationship	Social-related females	<i>n</i>	<i>n</i> nests	Solitary combined	<i>n</i>	<i>n</i> nests	df	<i>t</i>	<i>P</i>		
Female offspring	0.74 (0.68–0.81)	24	3	0.64 (0.54–0.74)	63	24	84.5	1.7	0.1		
Social females	0.71 (0.60–0.81)	24	13	–	–	–0.81 (0.67–0.97)	11	7	22.7	–1.3	0.2
Male–female offspring	–	–	–	–	–	–0.19 (0.07–0.31)	8	4	–	–	–
							df	<i>F</i>	<i>P</i>		
Female–male offspring/males–females	0.34 (0.31–0.37)	21	3	0.26 (0.22–0.30)	88	10	2, 128	2.1	0.13		

Mono solitary = singly mated solitary nests; Multi solitary = multiply mated solitary nests; Social-related females = all social nests except those containing unrelated adult females; Solitary combined = all solitary nests. Female–male offspring/males–females comparison represents the average pairwise relatedness between brothers and sisters in social and solitary nests, and between adult males and females in bisex nests. *t*-Statistics were calculated from a Welch's *t*-test for unequal variances and sample sizes and *F*-statistics were calculated with an ANOVA. Significant *P*-values are highlighted in bold.

evidence for multiple mating in 24% of solitary nests (9.4% of all genotyped nests) which translated to decreased relatedness between female offspring (Table 2). In the absence of behavioral observations, it is not possible to conclude whether social pairs in this study were daughters of multiply mated females, mother–daughter pairs, or both. Mother–daughter pairs could form if first-brood female offspring did not disperse and remained within her natal nest. This mother–daughter scenario would also translate into increased relatedness between social secondaries and male and female offspring ( $r = 0.5$ ) compared to full sister pairs where secondaries are related to offspring by 0.375 (Trivers and Hare 1976).

*Ceratina australensis* social secondaries have reduced ovarian development compared to primary females (Rehan et al. 2010, 2015) and so do not likely reproduce. We genotyped three social nests containing female offspring (Table 2;  $n = 24$  pairwise comparisons) which were likely full sisters ( $r = 0.74$ ) inferring that offspring were sired from a single, singly mated female. This was similar to the observed sister relatedness in the one social nest containing 3–4 females genotyped (Table 2;  $n = 6$  pairwise comparisons). Females in social nests with 3–4 females were highly related ( $r = 0.83$ ) and WW scores from these nests, as well as the relatedness for daughters ( $r = 0.69$ ), indicate that only one female was foraging and reproducing. Remaining at the nest to protect against brood parasitism has been cited as a probable mechanism for the fixation of traits related to limited dispersal in *C. australensis* (Rehan et al. 2011, 2014b) though we found no evidence for increased brood survivorship in social nests. As we did not rear offspring to adulthood as has been done previously to infer survivorship (Rehan et al. 2014b), it is possible that our rough estimate of survivorship did not capture this potential benefit of social nesting. The low frequency of social nesting in *C. australensis* (Table 1; Rehan et al. 2010) coupled with the low fitness of social secondaries (Fig. 3; Rehan et al. 2014b) indicates that the potential benefits do not outweigh the costs of forgoing reproduction.

While long-term bet-hedging against parasite attack in years or locations of high parasitism might explain the retention of social traits in seemingly low fitness years (Rehan et al. 2011, Kennedy et al. 2018), we cannot discount the potential role that cryptic in-nest behaviors may play in social nest formation. For instance, maternal manipulation of pollen sources leads to social nest formation in the



**Fig. 3.** Fitness of each reproductive strategy. Reproductive strategies were each significantly greater ( $P < 0.001$ ) than nonreproductive strategies but did not differ from each other. Male fitness was equal to zero.

North American *Ceratina calcarata* (Lawson et al. 2016). Maternal manipulation of pollen sources leads to the emergence of a small (in comparison to the mother) daughter who can then be coerced into helping at the nest (Rehan et al. 2014a). However, in *C. australensis*, social pairs are age and size-matched sisters (Rehan et al. 2014b). As such, in-nest behavioral observations are necessary to better understand the proximate mechanisms involved in social nest formation.

### Diploid Male Production

Two males from bisex nests were diploid in SA, the most genetically homogenous population (Dew et al. 2016, Oppenheimer et al. 2018). The presence of diploid males and bisex nests should not be conflated together, as bisex nests were discovered in both SA and QLD, while diploid males were only discovered in SA. SA likely represents the range edge for *C. australensis* and the probability of encountering a heterozygous individual was lowest in SA (Oppenheimer et al. 2018). The expected frequency of diploid males in SA was 8.6% which is low to moderate compared to previous estimates of male diploidy from previous studies of bee population genetic studies (Kukuk and May 1990, Packer and Owen 1990, Roubik

et al. 1996, Boff et al. 2014, Soro et al. 2017). As diploid males are thought to be effectively sterile and have reduced copulatory and/or sperm viability (Cook 1993), though there are exceptions (Cowan and Stahlhut 2004, Elias et al. 2009), females which mate with diploid males are thought to produce unfertilized eggs resulting in a male-biased brood (Cook and Crozier 1995). Interestingly, Dew et al. (2018) found SA to be characterized by a nonbiased offspring sex ratio of the first-brood cell position compared to QLD and VIC, which were highly female-biased. As a female bias in the first-brood cell is associated with maternal manipulation of pollen provisions in other *Ceratina* (Sakagami and Maeta 1977, 1984; Rehan and Richards 2010; Lawson et al. 2016), the observed sex ratio in *C. australensis* from Dew et al. (2018) is possibly the result of maternal sex allocation as opposed to the result of matings with diploid males. Increased rates of homozygosity, as were observed in this study, can produce males from fertilized eggs, and thus the even sex ratio in SA may be a by-product of increased matched matings between individuals carrying similar sex alleles (Cook and Crozier 1995).

### Social Evolution

Male helping behavior could potentially evolve under rigid circumstances (i.e., strong investment in male-biased broods); however, male and female behavior should not evolve within the same population (Bartz 1982). *Ceratina australensis* male cohabitation was rarely observed in one season in 2017, zero times in 2015–2016 (Table 1), and never previously 2007–2010 (Rehan et al. 2014b). Bisex nesting is likely not very adaptive for this species, but may occur on the rare occasion when males delay dispersal until before the end of the reproductive period. It is generally thought that most ceratinine bees mate in spring, though it is likely that the bivoltine *C. australensis* mate postemergence each brood period as first-brood females begin nesting upon emergence (Rehan et al. 2010). In the Japanese congener, *C. flavipes*, bees mate before and after hibernation and males survive the entire reproductive season (Kidokoro et al. 2006). Future long-term studies are needed to examine the sex ratio of brood in both reproductive periods of *C. australensis* to better understand the mating biology of this species. If a population wide sex-biased brood ratio occurs during the first reproductive period but not the second (Dew et al. 2018) or vice versa, this could affect the potential number of mates available for females at a given time, altering the cost-benefit ratios of mating with relatives and dispersing to found new nests (Quiñones and Pen 2017).

The factors related to mating in Hymenoptera are understudied due to the cryptic nature of mating; it is rarely observed and males are rarely studied (Paxton 2005). However, the timing of mating (before or after diapause) and the cues that are related to the onset of mating and the number of mates (cuticular hydrocarbons, sexual maturity of males and females) are related to factors such as dispersal and relatedness between individuals. These factors are often cited as precursors to social evolution and thus mating biology remains as an important, yet relatively understudied component in hymenopteran sociobiology. Here we present the curious case of male nesting behavior in the Australian small carpenter bees. Our data did not find an adaptive significance to this behavior and in fact tolerance may be detrimental to the overall fitness of females that allow males to stay in the nest. Future long-term studies are needed to determine the ecological circumstances and fitness consequences across generations. Social insect research typically focuses on females and we encourage future attention to note male behavior in life history studies.

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