

1 **Unrelated males in colonies of facultatively social bee**

2 **Short title: Unrelated males in bee colonies**

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8

9 **Abstract**

10 Colonies of social Hymenoptera are usually groups of closely related females, in which the dominant
11 female(s) is specialized for reproduction and subordinate females care for immature offspring. Kin
12 selection is thought to be the main factor that supports social cohesion. We have discovered a simple
13 society of the bee *Ceratina chalybea* with an average of 4.68 colony members that cannot be
14 maintained by kin selection alone. These colonies consisted of old reproductive female, young adults
15 and provisioned brood cells. About half of young adults are unrelated to the old female, and almost
16 all of the young adults are male. The old female provisions new brood cells, while continuing to feed
17 young adult offspring. As young adults do not perform demanding or risky activities, they incur little
18 or no cost, but they do benefit from the food they obtain from the old female.

19

20 **Introduction**

21 Cooperation between individuals is one of the most interesting biological phenomena. Several
22 mechanisms of cooperation have been proposed [1–4], but it is thought that kin selection is the main
23 mechanism driving the organization of societies [5–7]. This theory claims that the spreading of alleles
24 through one's own reproduction is equivalent to the spreading of alleles by related individuals [6,8].
25 The strength of kin selection is strongly influenced by relatedness – any help provided to another
26 individual is only beneficial if the cost of helping that individual is lower than the benefit of the help
27 received multiplied by the relatedness between the individuals [6,9]. Another mechanism of
28 cooperation that has been postulated is based on reciprocity: the cost of helping is compensated by
29 the predicted future benefit [1,3]. Alternatively, cooperation may be a by-product of selfish
30 behaviour [4]. Recently, the plausibility of group selection has been disputed – sacrificing individuals
31 for group benefit can exist as an adaptive feature [5,10,11].

32 Colony members in almost all social insects are related [7,9,12]. Cooperation of unrelated individuals
33 occurs under specific conditions and some derived states [13,14]. Pleometrosis (nest founding by
34 multiple females) is one of the most common situations in which unrelated members of a colony
35 cooperate [13,15]. In many wasp and bee societies, some proportion of drifting workers have been
36 recorded [14,16]. These workers originate from foreign colonies and are therefore not related to the
37 original nest members [14]. Unlike eusocial insects, unrelated helpers are common in cooperative
38 breeding vertebrates [2].

39 Societies of the eusocial Hymenoptera are mostly composed of females because the queens and
40 workers are female [17,18] and most males die soon after mating, not participating in the life of the
41 society [18–20]. Exceptions, such as male participation in care, are rare among eusocial societies and
42 male helpers usually have only a minor role in colony life [21–23].

43 The traditional division of labour in hymenopteran societies is between the queen, who performs
44 most or all egg-laying, and the workers, who are responsible for other tasks, especially food
45 provisioning [18,24]. However, in small societies that are composed of one reproductively dominant
46 female and one, or a few other females, a different type of task division is possible. The dominant
47 female can perform egg-laying as well as food provisioning, with the reproductively subordinate
48 female(s) performing nest guarding and other tasks in the nest [25]. This system of provisioning by
49 the reproductively dominant female is typical for Xylocopinae bees [25,26].

50 Here, we examined social nests of the small carpenter bee, *Ceratina chalybea*. Small carpenter bees
51 from the genus *Ceratina* make their nests in broken twigs with soft pith [27]. The female excavates a
52 burrow in the pith and later provisions the brood cells [28,29]. After completion of cell provisioning,
53 the female usually guards the offspring until they reach adulthood [29–31]. Subsequently, she
54 provisions newly emerged offspring with pollen and nectar [31–33]. Although most temperate
55 *Ceratina* are solitary [30,34], they belong to the Xylocopinae family, which is ancestrally facultatively

56 social, with obligate solitary nesting being a derived state [35]. Social nests of *Ceratina* contain a few
57 females, usually two but sometimes as many as four [36,37].

58 Until now, *C. chalybea* was thought to be a solitary bee [30]; however, here we present the first
59 evidence of social nesting in this species. In contrast to most *Ceratina* bees, the females of this
60 species did not obligately guard their offspring until adulthood, rather females had two alternative
61 strategies – either guarding or abandoning the nest after provisioning of brood cells is finished [30].
62 Clearly, mothers can only perform provisioning of young adults in guarded nests and only these nests
63 can develop into eusocial colonies. Here, we describe the social nests of *C. chalybea*. Moreover, we
64 tested relatedness between colony members. We try examine possible costs and benefits for
65 reproductive dominant and subordinate colony members.

66

67 **Methods**

68 **Study site**

69 We performed field research at the Havranické vřesoviště (coordinates 48°48'32.6"N 15°59'33.6"E)
70 location, near the village of Havraníky, in Podyjí National park. This location is situated in the
71 Southern Moravian region of the Czech Republic. The main experiments were performed in the years
72 2015 and 2017, but additional data are also presented from the years 2013 and 2018. Office of Podyjí
73 National park permitted this research.

74

75 **Preparation of nesting opportunities**

76 We studied nests made in artificial nesting opportunities. The nesting opportunities were made from
77 cut twigs with pith, from the following plant species: *Solidago canadensis*, *Helianthus tuberosus*, or
78 *Echinops sphaerocephalus*. Twenty twigs were tied together into a sheaf and fixed to the ground with

79 a bamboo rod. We distributed more than 20,000 nesting opportunities (1000 sheaves) each year. In
80 2013 and 2015, we collected nests directly from the nesting opportunities. In 2017 and 2018, some
81 nests were collected directly from sheaves at nesting site and other nests were taken from original
82 sheaves and transport to study plot for observation. Nests were collected and dissected after the
83 observation period.

84

85 **Nest dissection**

86 We collected nests in the evening (after 19:00) to ensure that all inhabitants would be present inside
87 the nest. We stored nests in a fridge between collection and dissection. We opened nests using a
88 knife or clippers, and for each nest we recorded the presence of all adults and non-adult juveniles
89 (eggs, larvae and pupae). For adults, we noted sex and age (parental vs filial generation). The age of
90 adults was easily recognized because adults of the parental generation had extensive wear to their
91 wings. All nests had only one old female. For non-adult juveniles, we recorded the stage and position
92 of its brood cell in the nest. We also recorded the number and position of any empty cells (cells
93 without provisions or offspring, [30]). We measured the length of the nest. We distinguished
94 between new nests and reused nests. Reused nests had adult excrements in the lower portion and
95 unsettled fillings below newly provisioned brood cells (Fig.1). For reused nests, we measured the
96 length of the actively used portion (from the lowest newly provisioned brood cell to the nest
97 entrance). Most of our analyses are based on nests in the late phase of the nesting season, between
98 July 15th and August 15th each year).

99

100 **Classification of nest stage**

101 We only used active brood nests for our analyses. Active brood nests were an outermost brood cell
102 containing a pollen ball or an egg; therefore, all nests that were currently provisioned. We

103 distinguished two types of nests: solitary nests and social nests. Solitary active brood nests
104 contained only a mother and the provisioned brood cells, no young adults. Social nests usually
105 contained a mother and always had at least one young adult in addition to the provisioned brood
106 cells. All together, between July 15th and August 15th we collected 28 social active brood nests (19 in
107 2015 and 9 in 2017) and 39 solitary active brood nests (19 in 2015 and 20 in 2017). We also classified
108 some nests as full brood nests. Full brood nests were those in which brood cell provisioning had
109 already been completed (the innermost brood cell contained a larva or pupa). Adult offspring are not
110 yet present in these nests, or if they are present, they have not crawled through the nest partitions.

111

112 **Observation of social nests**

113 We transported the nests used for observation to special study plots. Transport of these nests was
114 performed in the evening to ensure that all inhabitants were inside the nest. Each study plot
115 contained 24 nets. Here, we only present the results from social *C. chalybea* nests; however, we
116 observed these nests along with nests of other stages and species. Each nest was observed for one
117 observational day, between 8:00 and 16:00 CEST on days with suitable weather. Each plot was
118 observed for the entire time by at least one observer but most of the time there were two observers
119 present. We marked foraging bees with an oil marker (Uni Paint) on the abdomen. We recorded the
120 departure and arrival of foraging bees and noted when adults only departed from a nest (did not
121 return) or newly arrived to a nest. It was necessary to cover the nest entrance with a transparent cup
122 after every arrival of a bee so that the subsequent departure could be observed, since departure is
123 usually very fast. This allowed us to verify whether the bee had been marked previously or if not, to
124 mark it. Nests were dissected after their observational day. We performed this experiment in 2017.
125 We successfully observed 4 social nests of *C. chalybea* per observational day. Two other nests were
126 observed for only a partial day, due to inclement weather conditions.

127

128 **Analysis of relatedness between individuals in the nest**

129 We previously developed microsatellites for *C. nigrolabiata* [38], which we also used for the analyses
130 of *C. chalybea*. We used the Chelex protocol for DNA isolation. We isolated DNA from the whole body
131 of eggs and larvae, or the abdomen of pupae and adults. We added 4-8 μ l proteinase K and 50 μ l of
132 10% Chelex suspension in ddH₂O to dried samples. We mixed the suspension and heated it to 55°C
133 for 45 min, then to 97°C for 8 min in a thermocycler (BioRad). We centrifuged the samples and froze
134 the supernatant for further use.

135 We used the Type-it Multiplex PCR Master Mix (Quiagen) for multiplex PCR according to the
136 manufacture's protocol. We used primers for ten microsatellite loci ([38], SI Appendix) at a
137 concentration of 0.05 μ mol/l. We used the following settings for PCR: 95°C for 15 minutes; 30 cycles
138 of 94°C for 30 s, 60°C for 90 s, 72°C for 60 s; and finally, 60°C for 30 min.

139 We mixed 0.8 μ l of PCR product with 8.8 μ l of formamide and 0.4 μ l of marker Liz 500 (Applied
140 Biosystems). We heated the mixture to 95°C for 5 min and let it cool down. Fragmentation analysis
141 was performed on a 16 capillary sequencer at the Laboratory of DNA sequencing at the Faculty of
142 Science, Charles University. We used GeneMarker1.91 software (SoftGenetics, State College,
143 Pennsylvania, USA) for the identification of alleles. Usually, this software correctly identified alleles;
144 however, sometimes manual correction of the size scanner and identification of alleles was
145 necessary.

146 From the 10 loci we used, 9 provided successful products and all were polymorphic, although
147 polymorphism was highly variable between loci. Loci had between 2 and 33 alleles. Allele frequencies
148 are summarized in [38]. We tested relatedness in 12 social nests. All these nests were collected in
149 2015. Together, we analyzed 12 old females, 21 non-adult juveniles (15 females and 6 males) and 52
150 young adults (5 females and 47 males).

151

152 **Analysis of relatedness between mother and young adults**

153 In social nests, we used two methods to determine if young individuals in the nest (young adults and
154 also non-adult juveniles) are related to the old female. Primarily, we manually compared the
155 genotypes of the old female and offspring to determine their loci compatibility. Male offspring
156 should contain only alleles that the old female has. Female offspring should share at least one
157 identical allele with the old female at each locus. We counted the number of loci that had alleles that
158 were incompatible with the maternal genotype. We also assessed the relatedness of young adults
159 and offspring in Kinship software [39] using the following analysis: Kinship analysis, relatedness
160 option (Pairwise relatedness: Kinship). As offspring could have a coefficient of relatedness as high as
161 0.5 and unrelated individuals could have a relatedness coefficient as low as 0, we used 0.25 as the
162 cut-off for related individuals. All offspring below this cut-off were considered unrelated to the old
163 female.

164

165 **Testing the maternity of young adult females**

166 To determine the compatibility of young adult females with possible offspring (non-adult juveniles),
167 we counted the number of incompatible loci – loci in the offspring that only had alleles that could not
168 have been inherited from the young adult. We compared the number of loci with incompatible loci
169 between non-adult juveniles and young adult females with the number of incompatible alleles
170 between non-adult juveniles and old females. We also compared the relatedness calculated by
171 Kinship software between non-adult juveniles and young adult females with the number of
172 incompatible loci between non-adult juveniles and old females.

173

174 **Testing the paternity of young adult males**

175 We used colony software [40] to test the paternity of young adult males (to the non-adult female
176 offspring) in social nests. Settings in Colony software were: Mating system – Female polygamy, Male
177 polygamy, without inbreeding; Species – Dioecious, Haplodiploid; Length of run – Very long; Analysis
178 method – FL; Likelihood precision – High. For other options, default settings were used. The locus
179 feature was set as all loci codominant. The probability of genotyping error was 0.01; the probability
180 of other errors (for example, mutations) was 0.001 for each locus. The old female was set as the
181 known mother.

182

183 **Statistics**

184 All statistical analyses were performed in R software [41]. To test the relationship between sociality
185 and nest reuse, we used Fisher's exact test. We tested for differences between social and solitary
186 nests with the year as a covariable (model equations were: feature of nest ~ year*sociality). For
187 length of nest, length of nest entrance, and number of brood cells, a linear model was used. For the
188 proportion of empty cells, a generalized linear model of binomial family was used. It was impossible
189 to test sociality and nest reuse in one model together because both factors were strongly correlated.
190 Therefore, we fitted a primary model with sociality (feature of nest ~ year*sociality) and a secondary
191 model with nest reuse (feature of nest ~ year*nest reuse). We compared the Akaike information
192 criteria (AIC) between models with sociality and models with nest reuse for all four tested features of
193 nests. We compared the relatedness between non-adult juveniles and young adult females with the
194 relatedness between non-adult juveniles and old females. We tested differences in the number of
195 incompatible loci by paired Wilcoxon test. We tested differences in relatedness by paired t-test.

196

197

198 **Results**

199 **Evidence for social nesting**

200 In the first part of the nesting season (until July 15th), we only observed solitary nests (2013 N=90;
201 2015 N = 22; 2017 N = 5; 2018 N = 19). None of these nests were reused from the previous season.
202 However, we did find social nests later in the *C. chalybea* nesting season, after the 15th of July. In
203 total, we found 28 social nests. After July 15th, half of the active brood nests we found in 2015 were
204 social nests (19/38, Table 1) and 31.03% (9/29) of the active brood nests we found in 2017 were
205 social nests. In 2013 and 2018, all dissected active brood nests were solitary (N=25, 2013; N=26,
206 2018). One social full brood nest was found in 2018.

207 On average, social nests contained 3.68 young adults (4.15 in 2015 and 2.66 in 2017, Table 2), and
208 the maximum number recorded was 9. Most of the young adults were male (89.32%, 92/103). The
209 reproductive female was present in most nests (82.14%, 23/28), but some social nests had been
210 orphaned.

211 Table 1. The number of solitary and social active brood nests in different years. Only nests collected
212 between 15 July and 15 August are included.

Sociality	Solitary nest			Social nest			Total	
	Nest reused?	No	Yes	Together	No	Yes		Together
2013		23	2	25	0	0	0	25
2015		15	4	19	3	16	19	38
2017		15	5	20	2	7	9	29
2018		23	3	26	0	0	0	26
Total		76	14	90	5	23	28	118

213

Table 2: Number of young adults in *C. chalybea* social nests

Year	2015	2017	Together
N nests analyzed	19	9	28
Average number of young adults	4.16	2.67	3.68
Maximum number of young adults	9	6	9
Minimum number of young adults	2	1	1
SD of young adults	2.09	1.41	2.00
Average number of young adults females	0.26	0.67	0.39
Average number of young adult males	3.89	2.00	3.29
Proportion of males in young adults	0.9367	0.7500	0.8932

214

215 **Comparison between solitary and social nests**

216 We compared the nest architecture of solitary and social active brood nests. There was a strong
217 association between social nesting and nest reuse: only 15.55% (14/90, Table 1) of solitary nests
218 were reused in comparison to 82.14% (23/28, Table 1) of social nests. This association was significant
219 for both years (Fisher exact test; 2015 – $p = 0.0002$, $N = 38$; 2017 – $p = 0.0140$, $N = 29$).

220 Solitary and social active brood nests did not differ in total nest length (linear model, $F = 0.81$ $p =$
221 0.3713 , $N = 67$), but they did significantly differ in the length of the actively used portion of the nest
222 (linear model, $F = 17.34$, $p < 0.0001$, $N = 67$). This result held true when we used nest reuse as the
223 explanatory variable instead of sociality (total nest length did not differ – linear model, $F=2.28$, $p =$
224 0.135 , $N = 67$; but the actively used portion of the nest did significantly differ in length – linear
225 model, $F = 43.85$, $p < 0.0001$, $N=67$). When we compared the AIC of both models, nest reuse was
226 better than sociality as an explanatory variable. There are fillings and excrement from previous
227 instances of nesting at the bottom of reused nests; therefore, the length of the effectively used space
228 is shorter (fig. 1).

229 We also found a difference in the number of brood cells provisioned. Social nests had significantly
230 fewer provisioned brood cells (linear model, $F = 7.21$ $p = 0.0093$, $N = 67$, Table 3). When we tested
231 nest reuse as an explanatory variable instead of sociality, the difference was also significant (linear
232 model, $F = 5.25$, $p = 0.0253$, $N = 67$); however, the model using sociality had a better AIC than the
233 model with nest reuse.

234 We also found a difference in the presence of empty cells. In almost all cases, brood cells were
235 separated by empty cells in solitary nests, but they were often adjacent in social nests. The
236 proportion of cells that were empty was significantly lower for social nests compared to solitary nests
237 (Binomial glm, deviance = 7.99, residual deviance = 27.73, $p = 0.0047$, $N = 67$, Table 3). When we
238 tested nest reuse as the explanatory variable instead of sociality, there was also a significant
239 difference (Binomial glm, deviance 5.97, residual deviance = 29.78, $p = 0.0146$, $N = 67$); however, the
240 model using sociality had a better AIC than the model with nest reuse.

241

242 Table 3: Comparison of social and solitary active brood nest characteristics. Only nests collected
243 between 15 July and 15 August are included.

Nest Type	Solitary active brood nests			Social active brood nests			All nests
	2015	2017	Together	2015	2017	Together	
Year							
Number of Nests Analyzed	19	20	39	19	9	28	67
Total Length of Nest – mean	24.53	23.32	23.91	24.31	26.83	25.12	24.41
Length of Nest Used – mean	22.97	20.98	21.95	14.23	17.30	15.21	19.13
Number of Brood Cells – mean	3.58	2.95	3.26	2.21	2.44	2.29	2.85
Proportion of Cells Empty – mean	0.52	0.56	0.54	0.33	0.38	0.34	0.46

244

245 Fig. 1: Reused nest of *C. chalybea*. From left to right there are: fillings with excrements of larvae,
246 excrements of young adults, an empty cell, the pollen ball of a brood cell currently being provisioned.

247

248

249 **Foraging activity in social nests**

250 We recorded high foraging activity in social nests, which were observed for a full day (mean = 16.5
251 foraging trips for a day, range = 12–20, N = 4 nests). In all cases, only one female performed regular
252 foraging activity. We also observed two additional nests per part of day. In one nest, foraging activity
253 was performed by reproductive female and in second nest no activity was recorded. We did not
254 observe any young adults performing foraging activity; however, we did note the emigration of
255 young adults who did not return to the nest (mean = 1.25 for a day and nest, range = 0–2, N = 4
256 nests). There was also one case of immigration by a young adult (observed entering the nest without
257 having previously departed).

258

259 **Relatedness in social nests**

260 **Relatedness between the old female and non-adult juveniles (eggs and larvae):** Software analysis in
261 Kinship software concluded that all non-adult juveniles were related to the old female. However,
262 manual comparison of genotypes showed, that one individual had one locus with one allele that
263 could not have been inherited from the old female. Therefore, non-adult juveniles had all loci
264 compatible with the old female's genotype (95.23%; N = 21, Fig. 2).

265

266 Fig. 2: Histograms showing relatedness between the old females and other members of the societies.
267 A) Number of incompatible loci between the old females and non-adult offspring (eggs and larvae),
268 N=21. B) Number of incompatible loci between the old females and young adults, N=52. C)
269 Relatedness between the old females and non-adult offspring (eggs and larvae), calculated using

270 Kingroup software, N=21. D) Relatedness between the old females and young adults, calculated using
271 Kingroup software, N=52.

272

273 **Relatedness between the old females and young adults:** Young adults were compatible with the old
274 female's genotype for all loci in 40.39% of cases (21/52, Fig 2); whereas, one locus disagreed with the
275 old female's genotype in 11.53% of cases (6/52). For the remaining cases (25/52), the genotyped
276 offspring had more than one locus that disagreed with the old female's genotype. Analysis using
277 Kinship software showed that 55.77% (29/52, Fig 2) of young adults were related to the old female
278 and 44.23% (23/52, Fig 2) were unrelated to the old female. Therefore, we assume that between
279 44.23% and 59.61% of young adults are unrelated to the old female.

280 **Relatedness between young adult females and non-adult juveniles:** Young adult females were only
281 present in 3 out of 12 nests that were genetically analyzed. We compared the relatedness between
282 young adult females and non-adult juveniles with the relatedness between the old female and non-
283 adult juveniles. There were 9 possible pairs of non-adult juveniles and young adult females for
284 evaluation. In 22.22% (2/9) of these possibilities, non-adult juveniles had all loci compatible with
285 maternity of the young adult female; whereas, one locus was incompatible in 33.33% (3/9) of cases
286 and more than one locus was incompatible in 44.44% (4/9) of cases. The genotype of non-adult
287 juvenile offspring was compatible with maternity of the old female in all loci. Therefore, non-adult
288 juveniles have significantly higher compatibility with the genotype of the old female than the young
289 adult females (paired Wilcoxon test, $V = 0$, $p = 0.0206$, $N=9$). Also, we found that the relatedness
290 calculated by Kinship software of non-adult juveniles was higher to old female than young adult
291 females (paired t-test, $t = 5.77$, $df = 8$, $p = 0.0004$, $N = 9$).

292 **Relatedness between young adult males and non-adult juveniles:** We tested the paternity of young
293 adult males using Colony software to determine their relatedness to the non-adult juvenile females.

294 This analysis showed that none of these offspring (N = 15) were fathered by any of the young adult
295 males. In all cases, the probability of paternity was less than 1%.

296

297 **Discussion**

298 **Sociality in *C. chalybea***

299 We suggest, that social nests of *C. chalybea* fulfill all three of the conditions for eusociality defined by
300 [24] and [18]: i) reproductive division of labour (only old females reproduce); ii) generation overlap
301 (adults of the parental and filial generations are present); and iii) cooperative brood care (adult
302 members of the colony cooperate in guarding, helping young offspring survive) are all present.

303 We confirmed the old female's dominant reproductive role by microsatellite analysis. In all but one
304 case, the genotypes of non-adult juveniles were compatible with maternity of the old female. We
305 suppose that the single locus incompatibility in this one individual is due to a genotyping error or
306 mutation. Young adult females were rarely present in social nests and their maternity is less probable
307 than the maternity of the old female. We also tested that young adult males do not father non-adult
308 juveniles. Therefore, we demonstrated that the old female strongly (probably exclusively) dominates
309 reproduction in social nests and it is highly likely that no other members of the colony reproduce.

310 The most disputable phenomenon is cooperative brood care. The old female performs all offspring
311 provisioning. We never observed other individuals performing regular foraging activity and we
312 recorded only an occasional emigration or immigration of young adults. We suppose that the
313 presence of young adults is beneficial for nest protection because unprotected nests of *Ceratina* bees
314 [30] and other nest-making social Hymenoptera [42,43] are vulnerable to invasion and destruction.

315 Social nesting was strongly associated with nest reuse in *C. chalybea*. Nest reuse is generally
316 considered a key factor for the development of sociality in *Ceratina* [36,37,44]. Although nest reuse
317 can be an important factor influencing nest structure in social nests, we showed that sociality itself is

318 a better predictor for the number of brood cells provisioned and the proportion of empty cells in a
319 nest. Therefore, we suggest that at least these aspects of nest use are directly affected by sociality
320 and not only the effects of nest reuse.

321

322 **Comparison with other insect societies**

323 The social structure of *C. chalybea* is unusual among social insects for several reasons: i) the presence
324 of young adult males, ii) an unusually high proportion of unrelated colony members, and iii)
325 reproductive subordinate individuals perform only nest guarding (not provisioning).

326 Almost all (89%) members of *C. chalybea* societies are males. This is interesting because in general,
327 males have a very minor role in the Aculeate Hymenoptera [17,19,20]. A few biparental species are
328 known, in particular crabronid wasps from the genus *Trypoxylon* [45,46] and *Ceratina nigrolabiata*
329 [38]. In almost all eusocial species, males are a small minority among the colony members and have a
330 marginal role in comparison to female workers [21,47]. An interesting exception is the crabronid
331 wasp, *Misrostigmus nigrophthalmus*, in which a high proportion of colony members are male, actively
332 participating in nest defense. They are even able to perform this task in the absence of female
333 helpers [22]. But it remains unknown why male participation in eusocial societies is so rare.
334 Phylogenetic constraints might be one explanation. The solitary ancestors of social species have
335 female care without male participation [17,18,20]. Males lack some morphological structures, such
336 as hairs for pollen collection and a sting, which are important for working effectively in eusocial
337 societies [20,48]. Uncommon male behaviours may arise from performing a task or a standard
338 behaviour with a different primary purpose. In our case, it is likely that males can help with nest
339 protection because they primarily block the nest entrance in self-defense. Regardless of how it
340 occurs, this behavior does lead to effective nest guarding.

341 We determined that about half of the young adults are unrelated to the old female in *C. chalybea*
342 societies. There exist various mechanisms for arising of insects societies composed of unrelated

343 members, such as pleometrosis [13,15], adopting of orphaned brood [49] or exchange of individuals
344 between neighbour colonies [14]. We can exclude the possibility of pleometrosis for *C. chalybea*,
345 because we never found more than one old female in the nest. Adoption of an unrelated brood is
346 possible because nest usurpation and brood removal do occur in *C. chalybea*; however, it is rare and
347 only occurs with orphaned nests [30]. Therefore, incomplete brood removal cannot explain the large
348 proportion of unrelated young adults in nests of *C. chalybea*. Thus, it is very likely that the unrelated
349 individuals in *C. chalybea* nests originate from neighbouring nests. We frequently observed young
350 adults emigrating from and immigrating to nests; therefore, we consider unrelated individuals to be
351 drifting from other nests.

352 The reproductively dominant (old) female in *C. chalybea* nests performs all foraging and
353 reproduction. Young adults are passive; they do not perform any regular foraging trips. This type of
354 division of labour is generally uncommon in eusocial Hymenoptera [18,24], but it is usual for
355 Xylocopine bees [25,26,37]. In *C. chalybea*, we found the direct opposite of classical queen-worker
356 task division: the *C. chalybea* old reproductive female performs all foraging trips and young adults
357 (reproductive subordinates) only perform guarding. This is different from Allodapine bees, where
358 multiple females commonly perform some foraging [50], and also from east Asian *Ceratina* of the
359 subgenus *Ceratinidia*, where dominance of reproduction is unstable [51,52]. A direct contradiction of
360 the classical queen-worker roles (foraging dominant, passive subordinate) does occur in *Xylocopa*
361 [26,51,53]. However, in *Xylocopa sulcatipes* societies with a larger number of adult members (about
362 6), foraging is performed by multiple individuals [53]. Therefore, we have probably found a
363 Hymenopteran society with the lowest proportion of foraging individuals.

364

365 **Benefits for young adults**

366 Subordinate members of insect societies usually benefit from indirect fitness [6,9,11]. However,
367 direct fitness benefits, such as the possibility of inheriting a dominant position [25,26,54] or direct
368 reproduction [55,56], can also be important.

369 Indirect fitness benefits only occur with non-zero relatedness between the donor and acceptor [6].
370 However, we have shown that about half of the young adults are unrelated to the old female.
371 Moreover, previous work indicates that *C. chalybea* has a multiple mating strategy [38], which is
372 unusual in simple hymenopteran societies [7]. Drifting individuals and multiple mating generate very
373 low relatedness between colony members. Half of the colony members, those that are unrelated,
374 cannot gain any indirect fitness benefit from helping. Furthermore, the other half of the colony
375 members, those that have non-zero relatedness to the colony's young adults, might only gain a small
376 indirect fitness benefit due to the lower productivity of social nests in comparison to solitary nests.

377 The possibility for nest inheritance is an important selection factor for the cooperation of unrelated
378 members in small insect societies [54]. Generally, in Xylocopinae bees, nest inheritance is probably a
379 very important driver [25,56]. However, in the case of *C. chalybea* this cannot be an important factor
380 for the retention of sociality, because most of the young adults are male and nest-loyal biparental
381 behaviour is unknown in this species [38]. Additionally, as we did not observe any case of nest reuse
382 from the previous season, therefore we suppose that each female will build new nest next year. For
383 these reasons, we can exclude benefits from nest inheritance as a reason for sociality in the case of
384 *C. chalybea*.

385 Reproductively subordinate colony members can sometimes directly reproduce in small
386 hymenopteran societies [54,57]. These individuals drifting to foreign nest frequently benefit from
387 direct reproduction and commonly reproduce more than domestic workers [55,58]. However, based
388 on our evidence, it is clear that the old female strongly dominates reproduction in *C. chalybea*
389 societies and reproduction by young adults is negligible or zero. Therefore, direct reproduction
390 cannot be an important motivation for a young adult to stay.

391 Care of adult offspring is an unusual trait among insects [59], but it is common in Xylocopinae bees
392 [33]. It is likely that young adults remain in their nests because they benefit from the food provided
393 by old female. Long-term cohabitation between an old female and young offspring is a widespread
394 feature in *Ceratina* bees [29]. Many studies of solitary nests have shown that the mother provides
395 pollen and nectar for her young adult offspring [28,31–33]. We argue that social nests arise from
396 nests where mothers feed their adult offspring: first, the mother feeds mature offspring and then she
397 begins to provision new brood cells. However, this strategy can have a significant cost. As
398 provisioning of young adults continues along with providing for new offspring, the mother must
399 divide her resources between the new brood cells and adult offspring; therefore, the amount of food
400 that can be allocated for brood cell provisioning and thus the number of new brood cells is
401 decreased. We observed that the pollen ball in the outermost (open) brood cell, which was currently
402 being provisioned, had an atypical shape in some social nests. We suppose that this pollen ball is
403 partially eaten by young adults. Simultaneous provisioning of brood cells and feeding of young adults
404 has also been documented for *Xylocopa pubescens*. Maternal care of young adults is an important
405 benefit for them [26,60].

406 Cooperation between organisms is dependent on the costs-benefits ratio [6]. When little cost occurs,
407 little benefit is required to maintain stable cooperation. Young adults of *C. chalybea* do not perform
408 foraging, which is a very risky task for workers in most social insects [18,61]. It is likely that the
409 presence of young adults in *C. chalybea* nests has few costs, because it does not reduce their lifetime
410 reproduction. Females of *C. chalybea* [30] and also other temperate *Ceratina* species do not
411 reproduce before overwintering [29]. Males of temperate *Ceratina* bees survive through the winter
412 and usually mate in the season after overwintering [31,38]. Therefore, remaining in the nest probably
413 has little or no cost to future reproductive success and consequently, only a small amount of benefit
414 is required for young adults to remain.

415

416 **Benefits for the old female**

417 There was exceptionally low productivity in social nests of *C. chalybea*. In total, there were fewer
418 brood cells provisioned in social nests than in solitary nests. This differs from other social species,
419 where the overall productivity of social nests is either higher [43,62] or at least the same as solitary
420 nests [44,63]. Contrary to workers in large societies, young adults in *C. chalybea* nests did not leave
421 to perform foraging; rather, they stayed inside their nest. However, non-foraging individuals can be
422 beneficial for the society in other ways. It has been shown that the presence of guards in the nest can
423 be effective protection against pollen robbery by conspecific females in *Xylocopa* [26] or nest
424 usurpation [56].

425 In comparison to solitary nesting, social nesting decreases the risk of total nest destruction [56,64]. In
426 the case of *C. chalybea*, removal of the mother from completely provisioned solitary nests
427 significantly decreases the survival of offspring due to attack by natural enemies [30]. Therefore, the
428 presence of young adults can be a benefit because they are able to protect the younger cohort of
429 offspring. Young adults can serve the nest community through two mechanisms: i) reducing or
430 eliminating the trade-off between nest guarding and offspring provisioning, and ii) at least
431 temporarily, protecting the nest after the death of the mother. It has been shown that social nesting
432 allows for more effective foraging in multiple facultatively eusocial species [26,65]. We did not test
433 the effectiveness of young adult guarding experimentally; however, we did find a difference between
434 solitary and social nests in their architecture. In social nests, empty cells were significantly less
435 frequent than in solitary nests. Empty cells are thought to be an adaptation for protection against
436 parasite attack [66]. Therefore, in social nests, the presence of young adults can protect against
437 attack and the old female are able to reduce the number of empty cells, allowing more space for
438 provisioned offspring. We observed that young adults were present in some *C. chalybea* nests from
439 which the mothers had already vanished. As these young adults are located in the nest entrance,
440 they can protect the brood cells against potential intruders.

441 It is possible that there was low nesting productivity in social nests because a significant proportion
442 of pollen and nectar was consumed by the young adults and, therefore, could not be used to build
443 brood cells. As about half of the young adults are related to the old female, feeding of young adults
444 alongside brood provisioning can be beneficial for her reproductive success because this supports
445 their survival [32]. From the old female's view, social nesting can be interpreted as maternal care for
446 two cohorts of offspring simultaneously: a new cohort of offspring in the brood cells and an old
447 cohort of young adult offspring. However, it is unclear why the old female tolerates unrelated young
448 adults in the nest. One possibility is that it may be difficult to discriminate between alien and own
449 offspring. In *C. calcarata*, the mother can discriminate between nestmate and non-nestmate young
450 females [67]; however, overall aggression among individuals in mature brood nests is generally low,
451 and when it does occur, it is more often against nestmate than non-nestmate young females [67].

452

453 **Implications of *C. chalybea* natural history for social evolution**

454 Our observations support the view that benefits for subordinate colony members in small insect
455 societies are not, in many cases, primarily connected to inclusive fitness. It is possible for some
456 females to gain direct fitness benefits, as has been documented in some studies on Xylocopine bees
457 [25,26]. However, in the case of *C. chalybea*, the main benefits are not in the possibility of nest
458 inheritance, but in the extended care of mature offspring. The old female provides pollen and nectar
459 to feed young adults, which helps them survive. The old female tolerates young adults in the nest,
460 because this can provide the benefit of increased nest protection. Therefore, our study supports the
461 importance of mutualistic interactions in the evolution of the early stages of sociality.

462 As costs to young adults are low, small benefits are sufficient for the maintenance of sociality. We
463 suppose that young adults mainly benefit from the food provided by the old female. Young adults
464 can help with protection against natural enemies; however, their primary motivation for this is
465 probably passive (self-defense). Although the observed society fulfills the definition of eusociality

466 proposed by [18,24], the motivation for the behavior of colony members is mainly selfish. Therefore,
467 the society of *C. chalybea* is something between eusociality and a two-cohort maternal subsociality.
468 Unrelated young adults can be considered parasites, as they take food resources from the old female
469 Eusociality is ancestral state for all Xylocoinae bees with strict solitariness being a derived strategy [35].
470 The unusual social organization of *C. chalybea* has some traits in common with typical Xylocopine
471 social organization, especially the presence of unrelated colony members [56,68] and passive
472 reproductively subordinate individuals [25,51]; however, in the quantity of these features, *C.*
473 *chalybea* is extreme, even among species of the subfamily Xylocopinae. Furthermore, *C. chalybea*
474 society is unique in its inclusion of male colony members.

475 Here, we have shown that eusociality in bees can be maintained even when the relatedness between
476 colony members is very low and indirect as well as direct fitness benefits (i.e. the possibility of nest
477 inheritance) play small roles. In this case, eusociality is supported by specific natural-history traits
478 (i.e. feeding pollen and nectar to mature offspring and nest reuse). Thus, our results show that good
479 knowledge of natural history is important for interpreting social evolution.

480

481

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492

493 **Data Availability**

494 Dataset is available as SI material of this paper.

495

496 **Author contribution**

497 MM and JS designed the research; MM, DB and JS performed the research; MM analyzed the data;

498 MM wrote the initial draft of the paper; all authors commented and finalized the paper.

499

500 **Competing of interests**

501 The authors declare no competing interests.

502

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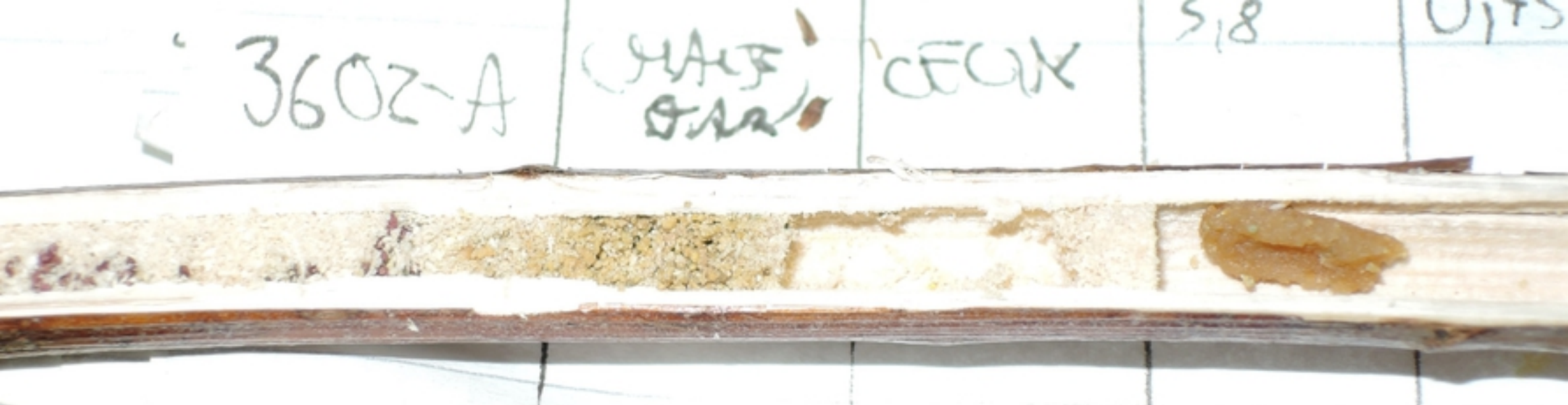
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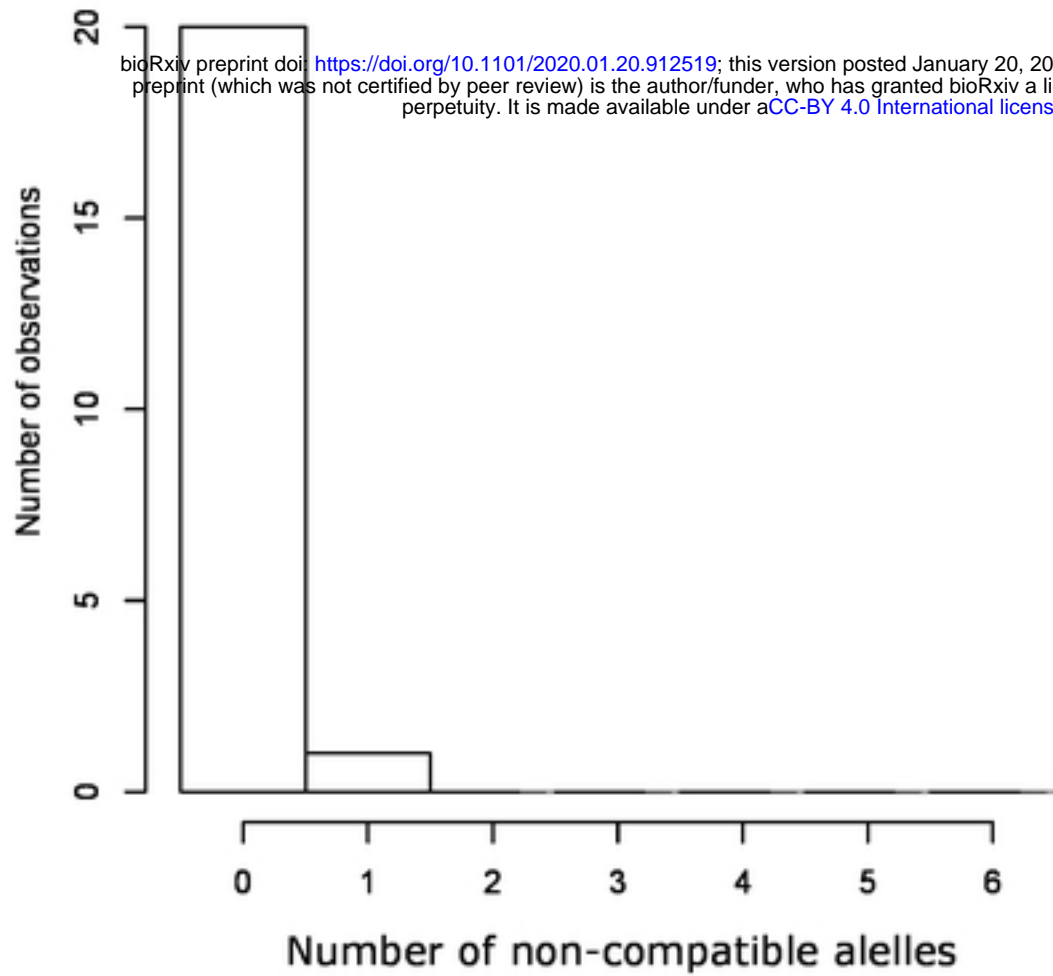
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Figure

Comparisons between old female and not-adult juveniles



Comparisons between old female and young adults

