

Research Article

Diploid Male Production of Two Amazonian *Melipona* Bees (Hymenoptera: Apidae)

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The diploid male has already been recorded for *Melipona* Illger, and herein, in *Melipona seminigra merrillae* Cockerell and *Melipona interrupta manaosensis* Schwarz. This paper was carried out at the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brazil. We produced and monitored 31 new colonies of *M. s. merrillae* and 32 new colonies of *M. i. manaosensis*. We sampled 2,995 pupae of *M. s. merrillae* and 2,020 of *M. i. manaosensis*. In colonies with a 1 : 1 sex ratio, male diploidy was confirmed by cytogenetic analysis and workers' behavior. We estimated 16 sex-determining alleles in *M. s. merrillae* and 22 in *M. i. manaosensis*. In colonies of *M. i. manaosensis* in a 1 : 1 sex ratio, workers killed the males and the queen that produced them soon after they emerged, as predicted. This behavior was not registered for *M. s. merrillae*, and sex ratios did not stay 1 : 1, indicating polyandry for this species.

1. Introduction

The haplodiploid mechanism of sex determination, or arrhenotoky, is a characteristic of Hymenopteran insects (ants, bees, wasps, and sawflies) and is widespread in invertebrate orders. It has independently evolved at least 17 times [1, 2]. In this sex determination mechanism, one fertile female (queen) lays fertilized and unfertilized eggs, which develop into diploid females and haploid males, respectively [1–6]. The diversity of sex-determining mechanisms that insects have evolved include heterogamy, haplodiploidy, paternal genome loss, X-chromosome elimination, and complementary sex determination (CSD) [5, 7–9]. Since Whiting [9], diploid males have been described for many arrhenotokous species [10–20]. In these species, the diploid male production (DMP) depends on the allelic composition at the gene *csd* (complementary sex determiner) [21, 22]. Under CSD, animals that are hemizygous at the *csd* locus become haploid males, whereas diploid individuals could de-

velop into females or males when they are heterozygous or homozygous, respectively. The production of diploid offspring in a 1 : 1 sex ratio occurs between males and females (queen) that share one allele at the *csd* locus [9, 16, 23, 24]. According to previous studies, when diploid males are viable, they are fully sterile. In many species, these males are killed by workers in the larval phase or soon after they emerge [10, 13, 25–29]. If viable and fertile, diploid males produce diploid sperm and lead to triploid female offspring, which would be a “reproductive-dead end” because these females are sterile [4, 30, 31]. The paradigm of genetic load associated with DMP was not confirmed in some vespids [6, 19, 32].

In colonies of eusocial insects, the negative effect of DMP leads to a loss of half of the worker force per generation [16, 33, 34]. Therefore, these species have evolved high polymorphism at the *csd* locus to avoid the impact of sterile diploid males [29].

In the parasitoid wasp *Habrobracon hebetor* (Braconidae), 9–20 sex-determining alleles were recorded [9, 35, 36].

For *Apis mellifera*, Adams et al. [23] estimated 18.9 sex-determining alleles and Tarpy et al. [37] reported 8–27. For stingless bees, 20 sex-determining alleles were estimated in *Melipona compressipes fasciculata* [13], 24 in *Melipona scutellaris* [16], 22 in *Melipona interrupta manaosensis* (this work), and 16 in *Melipona seminigra merrillae* (this work). In native and introduced populations of the fire ant *Solenopsis invicta*, Ross et al. [38] reported 115–120 sex-determining alleles.

Similar to the self-incompatibility loci in plants, the high polymorphism of the *csd* gene is maintained by a strong selection pressure [22, 23, 39–42]. If k is the effective number of sex alleles in a panmictic population, the probability of a matched mating is $2/k$, and the number of diploid individuals that is expected to be male is $1/k$ [23, 39, 40, 43]. Therefore, in natural populations, the expected frequency of diploid males is low [40], although inbreeding usually results in alterations.

Molecular studies in *Apis mellifera* showed the *csd* gene chromosome localization [44] and isolated and identified this primary signal of sexual development [24]. This gene has not yet been mapped or isolated in stingless bees, but diploid males have been recorded for some *Melipona* species [11, 13, 16, 25].

Since the number of mates increases the genetic variability and so the number of sex-determining alleles, females' mating frequencies, are an important parameter in studies of mating systems. Queens' mating frequencies in both solitary and social Hymenoptera, range from exclusively monandrous (queen mates once) [45–47] to extreme polyandry (queen mates more than six times) [45, 48–52].

Mating frequency in queens of bees is also variable. Most bee species are solitary with queens mating once, which is supported by chemical and ecological studies [53, 54]. However, many solitary bees mate multiply [48]. Thus, more studies on the mating system in the solitary bees are needed [53]. Studies on mating frequency in the genus *Bombus* (bumble bees) showed monandry for most species [55]. In the stingless bees studied so far, single mate seems to be a rule [45, 56, 57]. However, cases of mating with two males or more have been reported [16, 58]. Despite being rare in eusocial Hymenoptera, polyandry has been well documented in ants (genus *Atta*), wasps (genus *Vespula*) and in the advanced eusocial bees (genus *Apis*) [51]. The genus *Apis* evolved to extreme polyandry, with mating frequency and effective paternity extremely variable among species and in some cases within the same species [37, 59–61]. The lower levels of polyandry were recorded for *Apis florea* (queens mate with 5–14 males [59]), and the higher levels were recorded for *Apis dorsata* (queens mate with 47–102 males [62]).

Research on the sex-determination mechanism of *M. s. merrillae* and *M. i. manaosensis* was carried out, and diploid males were seen in both species. The genetic diversity was calculated through diploid male frequency. The workers' behavior in colonies producing diploid males (in a 1:1 sex ratio) was registered daily. The expected behavior of *Melipona* workers was based on previous studies [11, 13, 16, 25] and was validated in *M. i. manaosensis*. However, the same behavior did not occur in *M. s. merrillae*, according to this study.

2. Materials and Methods

Thirty-one new colonies of *M. s. merrillae* (Figure 1(a)) and thirty-two new colonies of *M. i. manaosensis* (Figure 1(b)) were produced. This was performed by the reproduction of 63 colonies in excellent conditions from the meliponary of the Grupo de Pesquisas em Abelhas (GPA) of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brazil, during 2007 and 2008.

The manipulated and reproduced colonies were in good condition when they presented large brood combs in different developmental stages, had access to provisions (honey and pollen pots surrounding the brood cells), and there was a massive population of adult workers (Figure 2(a)–(d)) [63]. The original colonies from which the additional colonies were derived were called the “mother” colonies. The bees were reared in standard boxes that facilitated the generation from the “mother” colony. As a result of the formation of this generation, one of the new colonies became an orphan (without a queen bee), which can last for a few days until a new queen is mated and established. The new queens were marked on the pronotum with a white spot of nontoxic ink (Figure 1(a)). After mating confirmation by the first oviposition, the subsequent egg laying was monitored. Each of the 63 new colonies was surveyed 40 days after the start of oviposition. To verify a 1:1 sex ratio, combs with 30 to 100 cells from the new queens' first brood were removed from the colony and reared in a temperature-controlled chamber at 28°C to complete the development into pupae. From these, 2,995 individual pupae of *M. s. merrillae* and 2,020 of *M. i. manaosensis* were sampled. Pupae of males and females were quantified to estimate the sex ratio. Data analysis was focused on matched mating or crosses that produced offspring in a 1:1 sex ratio. The goodness-of-fit was performed by a G-test [64]. The polymorphism at the sex-determining locus was estimated by Laidlaw's equation [16, 65] [$n = 2M(N + 1)/(H + 1)$], where n = sex allele number, N = sampled colonies number, H = number of colonies that segregate diploid male, and M = number of males that fertilized the queen. According to the technique described by Imai et al. [66], Francini et al. [67], in each colony with a 1:1 sex ratio, we carried out cytogenetic analysis of 20–30 males to confirm male diploidy and just 1–2% of the males analyzed cytogenetically were not diploid. In these colonies, the workers' behavior was observed daily and photo documented. We also randomly sampled colonies with other than a 1:1 sex ratio to perform cytogenetic analysis of males.

3. Results

Data of sex segregation and cytogenetic analysis confirmed diploid male production in both species (Table 1). Three of thirty-one *M. s. merrillae* colonies monitored presented offspring in a 1:1 sex ratio, while the same was verified in two of the thirty-two colonies of *M. i. manaosensis*. In these colonies, most males analyzed cytogenetically showed a diploid number of $2n = 18$ in *M. i. manaosensis* and $2n = 22$ in *M. s. merrillae*. We also observed diploid males in colonies with other than a 1:1 sex ratio. Assuming monandry (queen

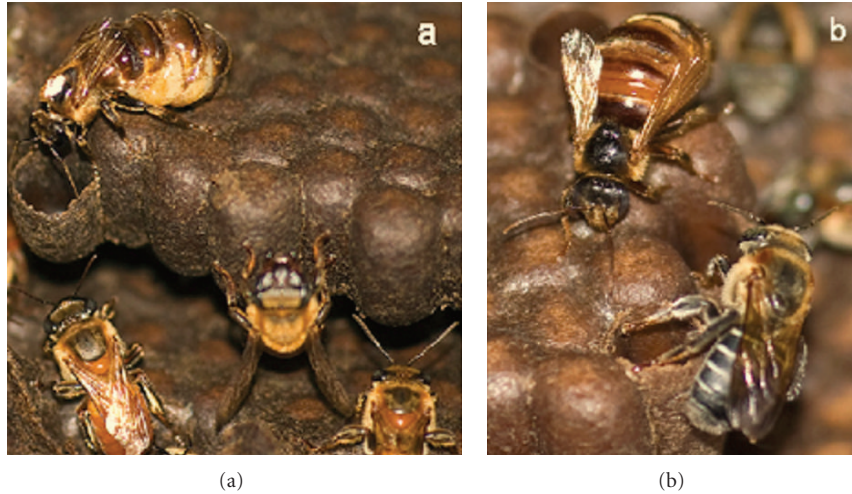


FIGURE 1: *Melipona seminigra merrillae* (a) showing the queen marked on the pronotum and workers showing the characteristic color of the scutellum in this subspecies; *Melipona interrupta manaosensis* (b) queen and worker characteristic colorations.



FIGURE 2: Colony conditions. (a) Colony in standard box, with large brood combs and surrounding pollen-pots; (b) colony inside tree-trunk with many brood combs, the age of developmental stage increases from upper to down, well evidenced by the darker color of combs in initial development; (c) workers of *Melipona seminigra merrillae* in honey-pots; (d) honey and pollen pots provision of the tree-trunk colony showed in (b) and many workers feeding on.

TABLE 1: Sex segregation of *Melipona seminigra merrillae* (colonies MSM) and *M. interrupta manaosensis* (colonies MIM).

Colonies MSM	♂	♀	Sex ratio	G-test	Colonies MIM	♂	♀	Sex ratio	G-test
01	06	89	0.06	S	01	00	74	0.00	S
02	00	74	0.00	S	02	00	36	0.00	S
03	07	55	0.11	S	03	00	63	0.00	S
04	09	78	0.10	S	04	00	31	0.00	S
05	00	60	0.00	S	05	00	53	0.00	S
06	00	63	0.00	S	06	00	69	0.00	S
07	14	55	0.20	S	07	00	81	0.00	S
08	00	69	0.00	S	08	08	32	0.20	S
09	06	82	0.07	S	09	05	78	0.06	S
10	13	21	0.38	S	⇒10	39	56	0.41	NS
11	09	33	0.21	S	11	00	65	0.00	S
12	07	46	0.13	S	12	00	84	0.00	S
13	00	52	0.00	S	13	01	64	0.02	S
14	27	66	0.29	S	14	00	57	0.00	S
15	00	81	0.00	S	15	01	62	0.02	S
16	87	00	1.00	S	16	13	59	0.18	S
17	00	70	0.00	S	17	11	51	0.18	S
18	35	76	0.31	S	18	00	88	0.00	S
19	04	55	0.07	S	19	03	66	0.04	S
⇒20	53	54	0.49	NS	20	00	78	0.00	S
21	03	80	0.04	S	21	00	46	0.00	S
22	16	43	0.27	S	22	03	57	0.05	S
23	16	84	0.16	S	23	00	53	0.00	S
24	86	13	0.87	S	24	00	58	0.00	S
25	03	56	0.05	S	25	00	64	0.00	S
26	22	61	0.26	S	26	00	51	0.00	S
⇒27	39	54	0.42	NS	27	00	57	0.00	S
28	77	52	0.60	S	28	01	62	0.02	S
⇒29	26	32	0.45	NS	29	00	48	0.00	S
30	3	97	0.03	S	30	00	63	0.00	S
31	12	48	0.20	S	31	00	79	0.00	S
					⇒32	21	29	0.42	NS

* Null hypothesis, sex ratio 1 : 1; G-test, critical values ($G = 3.841$; $DF = 1$; $P = 0.95$ and $\alpha = 0.05$); S: significant; NS: nonsignificant; ⇒ Colonies with a 1 : 1 sex ratio.

mates once) as predicted for Meliponini queens [16, 63], we estimated 16 sex-determining alleles in *M. s. merrillae* and 22 in *M. i. manaosensis*.

In *M. i. manaosensis*, the workers' behavior in colonies with a 1 : 1 sex ratio confirmed what was predicted for the *Melipona* genus [11, 13, 16]: workers killed both the diploid males (Figure 3(a)) and the queen mother (Figure 3(b)) that produced them as soon as the diploid males emerged. However, this behavior was not observed in *M. s. merrillae*.

To verify that the queen continued to produce diploid males, we sampled the second and the third brood combs in addition to the first in colonies that had a 1 : 1 sex ratio in the first brood comb. In *M. s. merrillae* colonies with a 1 : 1 sex ratio in the first brood, we found that this ratio was not maintained in the subsequent combs. We recorded a deviation in this ratio in the second and the third brood comb, both female biased and male biased (Table 2).

4. Discussion

The frequency of diploid male for the majority of Hymenoptera studied is an indicator of genetic diversity and its loss [18]. This is a parameter that should be highlighted in stingless bees, both for the sake of conservation and for beekeeping as an economic alternative [16, 42, 68] for the Amazon people. The viability of diploid males was described previously for three *Melipona* species. In all cases, the workers killed their diploid brothers and the queen mothers that produced them [11, 13, 16], as also documented here for *M. i. manaosensis* (Figure 3). We did not observe workers of *M. s. merrillae* killing their diploid brothers or their mothers. Thus, in *M. s. merrillae* the workers behavior in colonies that produce diploid males seems to contradict that recorded previously for *Melipona*.

Data indicated that the queens of *M. s. merrillae* had mated with two or more males. The deviation from a 1 : 1



FIGURE 3: *Melipona interrupta manaosensis*, the workers' behavior in colonies with a 1 : 1 sex ratio. Workers are attacking a male (a) and killing the queen (b) in the same colony at the same time.

TABLE 2: Variation of the sex ratio in colonies of *Melipona seminigra merrillae*. Queens that produced diploid males (a 1 : 1 sex ratio in the first brood comb) did not maintain this ratio in the second and third brood combs.

Colony	♂	♀	Sex ratio	G-test
20/D1	53	54	0.49	NS
20/D2	28	15	0.65	S
20/D3	19	99	0.16	S
27/D1	39	54	0.42	NS
27/D2	44	36	0.55	NS
27/D3	17	41	0.29	S
29/D1	26	32	0.45	NS
29/D2	33	65	0.34	S
29/D3	03	75	0.04	S

* Null hypothesis, in a 1 : 1 sex ratio; G-test, critical values ($G = 3.841$; $DF = 1$; $P = 0.95$ and $\alpha = 0.05$); S: significant; NS: nonsignificant; D1, first brood comb; D2, second brood comb; D3, third brood comb.

sex ratio along the different brood combs of the same queen (Table 2) seems to be evidence of polyandry [1, 4]. Additionally, we observed diploid males in colonies with other than a 1 : 1 sex ratio, which corroborated data in Table 1. Polyandry increases genetic variability, which is advantageous in a complementary sex-determination system [69]. An increase in mating frequencies leads the queen to produce diploid males at a frequency of $1/n$ of the population in the condition of panmixia [70]. Thus, polyandry can explain the variation in the sex ratio of the brood combs from the same queen, observed here in *M. s. merrillae*. Polyandry may be a good strategy evolved by *M. s. merrillae* to avoid the costs of the DMP [11, 13, 16]. This behavior is probably unique to *Melipona*, but more studies are necessary.

Despite the evidence here, the number sex-determining alleles was estimated under the assumption that queens are monandrous, according to the available information on *Melipona* [63]. Using Laidlaw's equation, estimates will be even higher with polyandry, so our estimates are likely low for *M. s. merrillae*.

Among the problems of the conservation of native bee fauna in Latin America, there is a need for basic information on taxonomy, genetics, ecology, and reproduction biology [70, 71]. The results presented herein should contribute to maintain local biodiversity associated with pollination by wild bees [68]. This work is an effort toward filling the lack of

indispensable knowledge for native bee conservation in the Neotropics, especially in the Amazon Basin.

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