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Original article

Testis length distinguishes haploid from diploid drones in *Melipona quadrifasciata* (Hymenoptera: Meliponinae)

Mara Garcia TAVARES*, André Southernman Teixeira IRSIGLER, Lucio Antonio DE OLIVEIRA CAMPOS

Departamento de Biologia Geral, Universidade Federal de Viçosa, 36571-000, Viçosa, MG, Brazil

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Abstract – Testes length in diploid and haploid larvae drones of *Melipona quadrifasciata* were investigated to ascertain whether it is a good character to distinguish haploid from diploid drones. Two groups of haploid and two groups of diploid drones were compared. The results show that diploid males have shorter testes than haploid males. Another interesting observation was that whereas diploid males from different groups had testes of similar sizes, haploid males produced by different queens had testes which differed significantly in size. It is suggested that measurements of the testes length could be used to determine ploidy and contribute to the understanding of the population biology of this and other related Hymenoptera species.

***Melipona quadrifasciata* / stingless bee / diploid drone / testicule**

1. INTRODUCTION

In Hymenoptera, sex is determined by haplodiploidy, a genetic system in which ordinary males are haploid and females are diploid. Diploid males, however, have now been detected in approximately 40 species including Diprionidae (Symphyta), Braconidae, Ichneumonidae, Apidae, Halictidae, Vespidae and Formicidae (Apocrita) (Cook, 1993; Carvalho et al., 1995; Crozier and Pamilo, 1996; Holloway et al., 1999; Noda, 2000). Experimental data based on the offspring of sibling matings demonstrated that the single locus multiple-allele system of sex determination (CSD) (Whiting, 1943) is operating in these species. According to this model heterozygotes at this locus are females, while homozygotes and hemizygotes are males.

While diploid male sterility is a general phenomenon, other aspects of their biology are more variable. For instance, in *Apis mellifera*, diploid male larvae are not viable because they are killed by the workers (Woyke, 1967). In *Bracon hebetor* these males have low viability and many fail to hatch from eggs (Whiting, 1943). However, when allowed to hatch artificially, the diploid male embryos are apparently as viable as haploid embryos (Petters and Mettus, 1980). Another species where diploid males are less viable than haploid males is the sawfly *Athalia rosae ruficornis* (Naito and Susuki, 1991). On the other hand, in *Bombus atratus* (Plowright and Pallet, 1979) and in *B. terrestris* (Duchateau and Marrien, 1995), diploid male larvae reach adulthood. Also in *Melipona quadrifasciata* (Camargo, 1982), *Lasioglossum zephyrum* (Kukuk and May, 1990), *Solenopsis invicta* (Ross and Fletcher,

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* Corresponding author: mtavares@mail.ufv.br

1985), *Diadromus pulchellus* (El Agoze and Periquet, 1993) and in *Neodiprion nigroscutun* (Smith and Wallace, 1971) diploid males have normal viability and develop to imagoes.

Diploid males resemble haploid males very closely in *Bracon* (Whiting, 1943) and in *M. quadrifasciata* (Camargo, 1979), but are clearly larger than haploids in other Hymenoptera species (Smith and Wallace, 1971; Woyke, 1978; Ross and Fletcher, 1985; Packer and Owen, 1990; Naito and Suzuki, 1991). However, in *B. terrestris*, diploid males are smaller than haploids, even if they are reared in the same brood in the same colony (Duchateau and Marien, 1995).

Testes size is a morphological feature that differs between haploid and diploid males of some bee species. In *A. mellifera* (Woyke, 1974) and in *B. terrestris* (Duchateau and Marien, 1995), the testes of diploid drones are smaller in comparison to those of haploid drones. Woyke (1973) showed that the testis of the diploid drones in *A. mellifera* have about half the number of seminiferous tubules, and these are only 42% as long as those of the haploids.

All these reports lead to several questions about the biology of diploid drones in Hymenoptera. In this study, we present results concerning size differences of testes of haploid and diploid drones in the stingless bee *Melipona quadrifasciata* Lepeletier. The relatively high survival rate of *M. quadrifasciata* diploid drones (Camargo, 1972) makes this species an ideal model for the understanding of different aspects of diploid male biology.

2. MATERIALS AND METHODS

Two groups of haploid and two groups of diploid drones were compared in the present study. Ten bright pink-eyed pupae were analysed in each group of drones, totalling forty drones. Pupae were chosen because after adult emergence the testes become smaller due to migration of the spermatozoa to the vasa deferentia and this could interfere in the results.

The haploid drones analysed were the progeny of two unrelated queens (Colonies 682-I and 565) mated in the laboratory according to Camargo (1972). Each virgin queen was mated with a sterile male, obtained by irradiation with a Cobalt pump (60.000r). The queens were maintained in Petri dishes together with young workers and once their abdomen size increased they were transferred to colonies from which the physogastric queens had been previously removed.

The first group of diploid drones analysed resulted from a mother-son mating (Colony 462). In this case, the queen mated with a sterile male as described above, and subsequently was inseminated by one of her sons. The progeny that resulted from this second mating consisted of diploid males and females (workers and queens) at the frequency of 1:1. This occurred because this progeny will carry only two sex alleles and half of the fertilised eggs will be homozygous for the sex alleles and develop into diploid males while half will be heterozygous at this locus and will develop into females (workers and queens). Note that there will also be a small number of haploid males resulting from non-fertilised eggs. Electrophoretic analyses, however, showed that the queen was heterozygous (Aa) for the esterase locus and that the son that mated with her possessed the recessive allele (a). Consequently, diploid drones and workers could be heterozygotes (Aa) or homozygotes (aa) at the frequency of 1:1 and the haploid males could be (A) or (a) also in the frequency of 1:1. No haploid drones of type (A) were detected at the time of the experiment and the proportion of heterozygous diploid drones (Aa) was similar to homozygotes (aa) drones. For this reason, it was assumed that all males that were being produced were diploid.

The second group of diploid drones analysed in this study was obtained from a brother-sister mating (Colony 740).

The drones were dissected in saline solution and both testes were removed. Length measurements were obtained from four seminiferous tubules of each testes with the software Image Pro Plus 4.0™. The measurements were taken by a person who could not discriminate, a priori, diploid from haploid males.

For each male the thorax width was measured in the same way, using the intertegular span as reference (Cane, 1987), to allow a comparison of the testes and body sizes. Data were submitted to the t-student statistical test at the level of 5% significance and to the Pearson correlation test.

3. RESULTS

Our results show that diploid male pupae have broader and shorter testes than haploid males (Fig. 1). The mean length of the right testis from the haploid drones of colony 682-I was 7.73 ± 0.91 mm and of the left testis, 7.57 ± 0.87 mm. For the haploid drones of

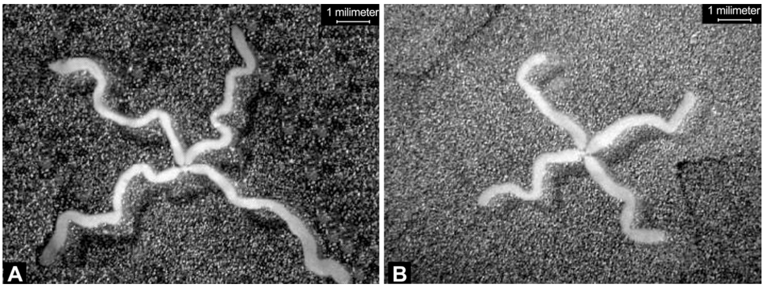

Figure 1. Testis of haploid (A) and diploid (B) *Melipona quadrifasciata* drone.

Table I. Morphometric data on testis length (mm) and thorax width (mm) in haploid and diploid males of *M. quadrifasciata.*

Groups of drones	Length of right testis (mean ± sd)	Length of left testis (mean ± sd)	Thorax width (mean ± sd)
1st Haploid (Col. 682-I)	7.73 ± 0.91^{a1}	7.57 ± 0.87^{a1}	2.72 ± 0.04^{a1}
2nd Haploid (Col. 565)	6.36 ± 0.99^{b1}	6.34 ± 0.82^{b1}	2.68 ± 0.08^{a1}
Mean	$7.05 \pm 0.96^{**3}$	$6.96 \pm 0.87^{**3}$	$2.70 \pm 0.03^{**3}$
1st Diploid (Col. 462)	4.66 ± 0.64^{A2}	4.69 ± 0.72^{A2}	2.86 ± 0.11^{A2}
2nd Diploid (Col. 740)	4.20 ± 0.60^{A2}	4.19 ± 0.72^{A2}	2.79 ± 0.13^{A2}
Mean	$4.43 \pm 0.33^{**3}$	$4.44 \pm 0.35^{**3}$	$2.83 \pm 0.05^{**3}$

Mean followed by the same letter did not differ statistically.
[1] comparative test of mean between haploid individuals; [2] comparative test of mean between diploid individuals; [3] comparative test of general mean between haploids and diploid individuals; ** $P < 0.01$.

colony 565, the mean length of the right testis was 6.36 ± 0.99 mm and that of the left testis was 6.34 ± 0.82 mm. Thus, the mean testes lengths of haploid drones in different colonies was significantly different (*t*-test, $P < 0.01$, Tab. I).

In the diploid drones of colony 462, the mean length of the right testis was 4.66 ± 0.64 mm and that of the left testis was 4.69 ± 0.72 mm. In the diploid drones of colony 740, the length of the right testis was 4.20 ± 0.60 mm and that of the left testis was 4.19 ± 0.72 mm. The difference between the mean of the testis length in the two diploid groups was not statistically significant (Tab. I).

Statistical comparison of the mean length of the right and left testis between haploid and diploid drones showed significant differences ($F = 116$, $P < 0.01$, Tab. I). Diploid and haploid drones could, therefore, be divided into two clearly distinct groups (Fig. 2).

The thorax width in haploid drones was 2.72 ± 0.04 mm in colony 682-I and 2.68 ± 0.08 mm in colony 565 (Tab. I). In the diploid drones thorax width was 2.86 ± 0.11 mm in colony 462; and 2.79 ± 0.13 mm in the colony 740. The mean of the thorax width in the first haploid group of drones was similar to the mean of the thorax width of the second diploid group, but the overall mean size of the thorax of the haploid drones (2.70 ± 0.03 mm) was significantly different from that of the diploid drones (2.83 ± 0.05 mm) ($F = 14.10$; $P < 0.01$).

There was a high correlation between testes length and thorax width in the diploid drones.

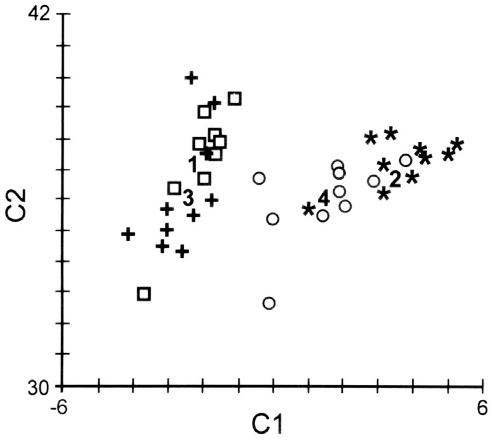

Figure 2. Haploid and diploid drone discrimination based on the measurement of the testis length. C1 and C2: scores of main components. Symbols "□" and "+" represent drones of the two haploid groups, while "*" and "o" represent drones of the two diploid groups. Numbers 1, 3, 2 and 4 are the respective centroids of each group represented.

However, this correlation was not observed (Tab. II) in the haploid drones.

4. DISCUSSION

In *Melipona quadrifasciata* sex is determined by a single multi-allelic locus. This implies that as a result of brother-sister mating 50% of the colonies will produce diploid males by homozygosity at the sex locus. The diploid males in such colonies make up 50% of the diploid brood. Mating involving a physogastric queen and one of her sons will also produce diploid males since the son shares one sex allele with his mother.

Physogastric-queen mating in stingless bees was first reported by Sakagami and Laroca (1963) in *Lestrimelitta ehrhardti*. The mating pair was found inside the nest during the manipulation of a colony. A few observations on mating and mating attempts with physogastric queens, all made under anomalous circumstances, have since been reported (Sakagami, 1982; Engels and Engels, 1988; Campos and Melo, 1990).

This is an interesting fact because stingless bees generally mate only once, according to DNA microsatellite data (Peters et al., 1999), a result in line with earlier sperm count in queen spermatheca and individual males and allozyme studies (Kerr et al., 1962; da Silva et al., 1972; Contel and Kerr, 1976; Machado et al., 1984). Paxton et al. (1999), however, verified that queen mating frequency varied between 1 and 3 in *M. beecheii* and 1 and 6 in *Scaptotrigona postica*.

In this study, a physogastric-queen that had been crossed with a sterile male was inseminated by one of her sons to produce diploid males, whose biology could be compared with haploid males.

Pupae of diploid males in *M. quadrifasciata* give rise to adult drones as viable as their worker sisters but our results showed that diploid male pupae have shorter testes than haploids.

Diploid and haploid drones could, therefore, be divided into two clearly separated

Table II. Correlation between the thorax width and testis length in *M. quadrifasciata*.

Groups of drones	Thorax X Right testis	Thorax X Left testis	Right testis X Left testis
1st Haploid (Col. 682-I)	0.31	0.32	0.77**
2nd Haploid (Col. 565)	0.58+	0.47	0.94**
1st Diploid (Col. 462)	0.95**	0.92**	0.89**
2nd Diploid (Col. 740)	0.61+	0.87**	0.80**

**, *, +: $P < 0.01$; 0.05 and 0.1, respectively.

groups when the mean testes length values were considered, showing that this character is quite useful for distinguishing haploid from diploid drones. Diploid males with smaller testes have also been found in *Apis mellifera* (Woyke, 1973) and *Bombus terrestris* (Duchateau and Marien, 1995). Furthermore, it was observed that whereas diploid males of different colonies had testes with similar sizes, haploid males produced by different queens had testes with significant length differences. The reasons for this difference, however, will only be determined after analysing additional colonies.

Our results also demonstrate that diploid *M. quadrifasciata* males have a wider thorax than haploid males. Larger size has been reported also for other diploid Hymenoptera males (Smith and Wallace, 1971; Woyke, 1978; Ross and Fletcher, 1985; Packer and Owen, 1990; Naito and Suzuki, 1991; El Agoze and Periquet, 1991).

Factors other than ploidy could explain differences in male size. Duchateau and Marien (1995) stated that differences in diploid male sizes are related to larval nourishment. For instance, in *Auglochlorella striata*, a eusocial mass provisioning bee, diploid males are often larger than haploid males, because the fertilized egg that gives rise to a diploid male would normally produce a female (Packer and Owen, 1990). In this case it is expected that a queen might respond to the food amount in a brood cell: with more food a diploid egg will be laid, so the diploid male larvae gets the same amount of food normally supplied to the female and therefore becomes larger. The same appears to be true for *M. quadrifasciata* where Bezerra (1995) observed that the cells in the centre of the comb possessed less food than those located in the periphery and that males arose preferentially in the centre whereas queens arose in the periphery. Thus, the grouping of males in the centre of the comb, as well as their production in pre-determined periods of the year, suggest that the physogastric queen of these bees is able to choose the progeny's sex. Again, in this case, diploid males arise from cells which should give rise to workers and thus are larger than haploid males.

As the thorax width can give a good estimate of the bee size (Cane, 1987), we tried to verified correlations between thorax width and testes length in *M. quadrifasciata,* since the testes length could depend on the male size. In the colonies analysed, however, we could only verify such correlation in diploid males. In haploid males, testes length was not related to body size, suggesting differences in the physiological processes between these drones.

Testes analyses of these drones have shown that diploid males produce less spermatozoa than haploid ones. These spermatozoa are produced later in diploid than in haploid males and they posses low mobility when stored in the seminal vesicle (Lino-Neto, unpublished data). These facts, associated with the short lifespan of the diploid male (Camargo, 1982) and probably the inability to fly, could contribute to its low fitness. In our laboratory, however, three diploid *M. quadrifasciata* males, each 16 days old, were able to mate with virgin queens. Unfortunately, these queens died a week after the mating and further analyses could not be done. Whether the spermatozoa of diploid males are not viable or unable to fertilise eggs is currently under investigation.

In conclusion, measurements of testes length are good evidence of the ploidy in *M. quadrifasciata* males and the study of diploid males is of special interest for the understanding of the reproductive biology of this and other related Hymenoptera species, which in turn, could contribute to our understanding of the genetic mechanism of sex determination in Hymenoptera.

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Résumé – La longueur des testicules distingue les mâles haploïdes des diploïdes chez *Melipona quadrifasciata* (Hymenoptera, Meliponinae). Chez les Hyménoptères, le sexe est déterminé par le système génétique de l'haplodiploïdie, selon lequel les mâles sont haploïdes et les femelles diploïdes. On a trouvé des mâles diploïdes, provenant de l'homozygotie au locus sexuel, chez environ 40 espèces d'Hyménoptères. Le taux relativement élevé de survie des mâles diploïdes de *Melipona quadrifasciata* fait de cette espèce un modèle idéal pour comprendre les divers aspects de la biologie des mâles diploïdes. Dans ce travail, nous présentons les résultats d'études comparatives portant sur la longueur des testicules de deux groupes de mâles haploïdes et deux groupes de mâles diploïdes. Les mâles ont été disséqués au stade de nymphe dans du liquide physiologique et les deux testicules ont été prélevés. Les mesures de la longueur ont été faites sur les tubes séminifères de chaque testicule avec le logiciel Image Pro Plus 4.0™. La largeur du thorax de chaque mâle a été mesurée de la même façon afin de pouvoir comparer la taille des testicules et la taille corporelle. Comme cela a été observé chez d'autres espèces, les mâles diploïdes de *M. quadrifasciata* ont des testicules plus petits que les mâles haploïdes (Fig. 1 ; Tab. I). Une comparaison statistique de la longueur moyenne des testicules entre mâles haploïdes et diploïdes a montré des différences significatives ($F = 116$, $P < 0{,}01$). Les mâles diploïdes et haploïdes peuvent donc être séparés en deux groupes distincts lorsque l'on prend en compte les valeurs moyennes (Fig. 2). Une corrélation élevée a été trouvée entre la largeur du thorax et la longueur des testicules chez les populations diploïdes, mais pas chez les haploïdes (Tab. II). La longueur des testicules des mâles haploïdes pourrait être un caractère important pour le développement des mâles normaux et leur fonction de reproduction. Pour cette raison, la longueur des testicules des mâles haploïdes ne serait pas corrélée à la taille corporelle. Le longueur des testicules est donc fort utile pour distinguer les mâles haploïdes des diploïdes.

***Melipona quadrifasciata* / abeille sans aiguillon / mâle diploïde / testicule**

Zusammenfassung – Unterschiedliche Länge der Hoden bei haploiden und diploiden Drohnen bei *Melipona quadrifasciata* (Hymenoptera: Meliponinae). Bei Hymenopteren wird das Geschlecht durch Haplodiploidie bestimmt, ein genetisches System, bei dem Drohnen normalerweise haploid und Weibchen diploid sind. Diploide Männchen, die durch Homozygotie am Sex Locus entstehen, wurden bei etwa 40 Arten bei Hymenopteren nachgewiesen. Die relativ hohe Überlebensrate von diploiden Drohnen bei *Melipona quadrifasciata* macht diese Art zu einem idealen Modell, um verschiedene Aspekte der Biologie der diploiden Männchen zu verstehen. Hier beschreiben wir die Ergebnisse einer vergleichenden Untersuchung der Länge der Hoden bei diploiden und haploiden Drohnen. Je zwei Gruppen mit haploiden und diploiden Drohnen wurden verglichen. Puppen dieser Männchen wurden in Kochsalzlösung aufgeschnitten und beide Hoden entnommen. Die Messung der Länge erfolgte bei 4 Samenschläuchen von jedem Hoden mit dem Computerprogramm Image Pro Plus 4.0™. Außerdem wurde von jedem Drohn die Breite des Thorax in gleicher Weise für einen Vergleich von Hodenlängen und Körpergröße gemessen. Wie bereits bei anderen Bienen beobachtet, haben diploide *M. quadrifasciata* Drohnen kleinere Hoden als haploide (Abb. 1; Tab. I). Ein statistischer Vergleich der mittleren Hodenlänge beider Drohnentypen ergab einen signifikanten Unterschied ($F = 116$, $P < 0{,}01$). Bei Zugrundelegung der Mittelwerte bildeten die diploiden und haploiden Drohnen zwei deutlich getrennte Gruppen (Abb. 2). Eine hohe Korrelation wurde zwischen der Breite des Thorax und der Länge der Hoden nur in der dipoiden Gruppe gefunden. Dagegen ergab sich keine Korrelation zwischen diesen Eigenschaften bei den haploiden Drohnen (Tab. II). Die Länge der Hoden bei den haploiden Drohnen könnte ein wichtiges Merkmal zur Entwicklung normaler Männchen und ihrer reproduktiven Funktion sein. Aus diesem Grund könnte die Länge der Hoden haploider Drohnen nicht mit der Körpergröße korreliert sein. Die verschiedene Länge der Hoden ist außerdem recht nützlich, um zwischen haploiden und diploiden Drohnen zu unterscheiden.

***Melipona quadrifasciata* / stachellose Bienen / diploide Drohnen / Hoden**

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