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Original article

Sexing of newly hatched live larvae of the honey bee, *Apis mellifera*, allows the recognition of diploid drones

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Abstract – In the honey bee, it is difficult to recognise the sex in first instar larvae. As workers and haploid drones develop in differently sized brood cells, they can be discriminated without sex inspection. However, because diploid drone larvae originate from fertilised eggs like workers, they hatch in brood cells of the same type and cannot be sampled according to cell size. In search of a reliable method of sexing live first instar larvae, we found that the contour and size proportions of the epiproct can be used for discrimination. The sex diagnosis based on these characters is carried out rapidly under a stereo microscope and allows the collection of pure samples of newly hatched diploid drone larvae from brood combs of inbred colonies.

***Apis mellifera* / sexing of live larvae / epiproct / diploid drones**

1. INTRODUCTION

The ovipositing honey bee queen measures the inner diameter of a brood cell with her forelegs (Koeniger, 1970) and lays fertilised eggs into small and unfertilised into large cells. Therefore, diploid female and haploid male larvae are found in worker and drone cells, respectively. However, diploid drone larvae are also found in worker brood cells because they develop from fertilised eggs (Woyke, 1965). Since they are eliminated by nurse bees within 36 h after hatching (Woyke, 1963), these males have to be discriminated from their sister females in neighbouring brood cells of the same size immediately after hatching to be used in any scientific studies. But sexing of living L1 larvae is not an easy task in honey bees or bumble bees (Duchateau and van Leeuven, 1990; Duchateau et al., 1994).

The male 'slit' and the 'greater pubescence' described by Kerr and Nielsen (1967) are difficult to detect, as is the

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estimation of a 'shorter epiproct'. However, by combining size differences and the contour of the epiproct, evident especially in slightly moving young larvae, a sexing of newly hatched live bee larvae is possible. This allows a method to sample live first instar diploid drones rapidly.

2. MATERIALS AND METHODS

Brood was used from normal and from highly inbred colonies of *Apis mellifera* in the apiary of the University of Tübingen. The inbred colonies were obtained by artificial insemination (Skowronek et al., 1995; Kühnert, 1991) of virgin queens with sperm of several brothers which resulted in about 25% of the progeny to be diploid males (Tab. II).

Brood combs with plenty of eggs were transferred into an incubator and examined for newly hatched larvae every 2 h. These unfed first instar larvae were collected and placed on a slide. Under a stereo microscope (Wild M 5) at 50 × magnification, the rear end of the larvae was examined with a micrometer. The slide was placed on wet filter paper in a petri dish on a warm plate of 35 °C. This environment avoids desiccation of the first instar larvae and keeps them moving, which is required for the sexing. Sex differences in the proportions and contours of the epiproct (the small 10th last abdominal segment, also called the anal segment) were detected by carefully observing about 200 freshly hatched female larvae from worker brood cells and haploid male larvae from drone brood cells.

The sexing efficiency was tested with a sample of live first instar larvae from a control colony. Workers and haploid drones were collected from respective brood combs and randomly placed on a microscopic slide. Their individual position was noted. Then the larvae were inspected under the microscope and sexed.

Furthermore, in 5 inbred colonies, the fraction of removed brood was compared with the proportion of sexed diploid drones. From each of the inbred colonies and from a control colony, a brood comb was taken and 103–233 cells with eggs were marked on a transparent sheet. After 4.5 days (3 days embryonic stadium plus 1.5 days during which the diploid drone larvae are eliminated), the number of empty cells was counted. The rate of removed larvae in the inbred colonies was corrected by subtraction of the background mortality as determined for the non-inbred control, resulting in the percentage of removed diploid drone larvae. For the same inbred colonies, in parallel the rate of males amongst the diploid larvae was determined by sexing some hundred live first instars.

To confirm the sex diagnosis of live larvae also by morphological evidence, about 150 diploid first instars, sampled from worker brood cells of an inbred colony, were first sexed and then fixed in Carnoy's solution and Congo red stained for subsequent analysis of the genital appendages according to Duchateau and van Leeuwen (1990).

In all collections of live diploid drone larvae, only those specimens were sampled in which the sex characters were clearly discernible.

3. RESULTS

3.1. Sex-specific properties of the abdominal tip in newly hatched bee larvae

During slow movements of living first instar larvae, a horizontal fold is formed in the upper part of the epiproct (Fig. 1). The proportions of the areas above and below this fold differ according to sex. In the male, a larger proportion of the epiproct, about one third to one half, is above the fold (a in Fig. 1A). In the female, only a minor

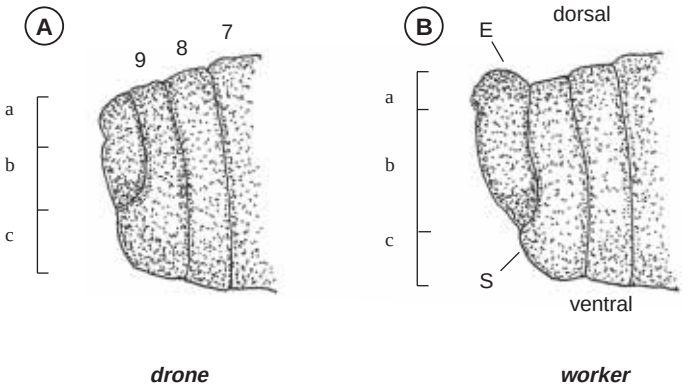

Figure 1. Rear end of first instar *Apis mellifera carnica* larvae. The sex diagnosis is based on size proportions and the contour of the (contracted) epiproct (Tab. I). 7 – 9 = abdominal segments, E = epiproct, S = sternum of the 9th segment.

portion, about one fourth, forms the upper part (a in Fig. 1B). In addition, only in the female this upper part of the epiproct reaches over the level of the dorsal contour of the penultimate 9th abdominal segment, which is not the case in the male (a in Fig. 1A). Moreover, in the female the lower part of the epiproct (b in Fig. 1B) is larger than the posterior sternum contour of the penultimate 9th segment, visible under the epiproct (c in Fig. 1B). In the male these two parts (b and c in Fig. 1A) are similar in size.

In summary, in the female (Fig. 1B) the part b is considerably longer than parts a and c, whereas in the male (Fig. 1A) part b is only a little longer. These sex-specific properties (Tab. I) become clear if measurements of the three parts in live larvae are taken using a micrometer, but most important are the proportions of the three parts, not their actual length.

Altogether a total of 3.310 live first instar larvae were sexed as females, haploid and diploid males by this new method. (Other members of our lab also succeeded with this approach.) No differences in the epiproct contours were found between haploid and diploid drones. Therefore, the sexing method described here can also be used to distinguish between diploid drones and their sister larvae, in inbred colonies lying in neighbouring worker brood cells. Subsequent tests were performed r to confirm this.

Table I. Sex differences for rapid distinction of living first instar honey bee larvae (Fig. 1).

♂	♀
(1) One third to one half of the epiproct is above the fold (a in Fig. 1A)	(1) about one forth of the epiproct is above the fold (a in Fig. 1B)
(2) The upper end of the epiproct does not loom over the penultimate (the 9th) segment	(2) the upper end of the epiproct looms over the penultimate segment
(3) The part of the epiproct under the fold (b in Fig. 1A) is about as long as the sternum of the penultimate segment (c)	(3) the part of the epiproct under the fold (b in Fig. 1B) is longer than the sternum of the 9th segment (c)

3.2. Confirmation of the sex diagnosis in first instar diploid drone larvae

The reliability of sexing newly hatched live bee larvae by the method described here was demonstrated by three tests.

1. The data of individually sexing 189 live worker and haploid drone first instar larvae of an artificially mixed sample were compared with the previous notes, resulting in a congruence of 100% of the male/female diagnosis.

2. The percentages of diploid drones as evidenced by emptied brood cells and in parallel by sexing live larvae were nearly identical for all the 5 inbred colonies (Tab. II), differing only between 1.1% and 6.2%.

3. By structural analysis of the genital appendages of diploid larvae, previously sexed alive, of 56 larvae diagnosed as males, in 53 (= 94.6%) this was correct. Of 98 larvae sexed as female, in 92 specimens (= 93.9%) the result was the same. In the other 3 and 6 larvae, respectively, the genital appendages were not visible and hence they were classified as undetermined. Thus, in 145 of 154 individuals (= 94.2%), the sex diagnosis of the live larvae was correct.

4. DISCUSSION

The newly hatched bee larva has a low status of external differentiation, lacking well pronounced sex-specific characters (Fleig and Sander, 1986). Only in the course of later larval instars the genital openings and appendages become visible (Nelson, 1915; Zander et al., 1916). In females the respective structures comprise the abdominal segments 7 to 9, whereas in males they are formed only in segment 9. Of the widely reduced last two segments 10 (and 11), merely the small epiproct is seen during all larval stages (Nelson, 1924), and its size (Kerr and Nielsen, 1967) and in particular its contour exhibit the only sex specific differences. As shown here, these characters can be used for sexing of newly hatched live larvae. With some experience, the diagnostic procedure described for this purpose allows a reliable sexing and, consequently, the discrimination of diploid drone larvae, thereby opening new perspectives of research with samples of diploid male larvae in bees. Since the sex diagnosis can be carried out quickly with living larvae, the separated specimens can be used for subsequent experiments. This allows, for the first time, the sampling of great

Table II. Frequency of diploid drones in inbred colonies. The percentages determined for young larvae removed from worker brood cells (data corrected for background cannibalism as recorded in the control colony) and sexing of newly hatched larvae resulted in nearly identical rates (bold figures).

Colonies	Number of marked brood cells with eggs	Rate of removed larvae [%]	Fraction of diploid drone larvae [%]	Number of sexed larvae	Fraction of diploid drone larvae [%]
Inbred					
No. 1	210	40.9	**33.2**	815	**34.3**
No. 2	233	29.2	**21.5**	148	**27.7**
No. 3	209	45.0	**37.3**	149	**34.2**
No. 4	231	44.8	**37.1**	1248	**33.4**
No. 5	158	33.7	**26.0**	554	**24.1**
Control	103	7.7	-	-	-

numbers of diploid drones from the brood of inbred colonies, facilitating especially analyses of the assumed chemical signals eliciting the cannibalistic behaviour of the nurse bees. In previous studies, mixed samples of diploid worker and diploid drone larvae had to be compared with pure samples of worker larvae (Bienefeld et al., 1994, 2000). In addition, in vitro rearing of series of sexed diploid drone larvae according to Woyke (1969) is now possible.

Additional proof for the reliability of the sexing of live first instar larvae is presented by our comparative tests. The rate of brood removal in the control colony was 7.7%. This background mortality corresponds well to data for non-inbred colonies (Woyke, 1977). Part of this mortality in normal colonies is due to the natural occurrence of diploid drones. E.g. with 20 sex alleles in a give honey bee population, the probability of diploid drone occurrence in a colony is 5%. We calculated corrected rates between 20% and 40% of diploid drone production by our inbred colonies with very good correspondence between the data per colony obtained by sexing of live larvae, the subsequent structural analysis according to Duchateau and van Leeuwen (1990), and the removal rates observed. Since in the three independent tests the sexing results were uniformly sustained, the new method can be considered reliable.

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Résumé – Le diagnostic du sexe chez les larves vivantes et fraîchement écloses de l'abeille, *Apis mellifera*, permet de reconnaître les mâles diploïdes. Chez l'abeille domestique (*Apis mellifera* L.) les larves de mâles diploïdes se développent dans des cellules de même type que celles des ouvrières. Il n'était pas possible avec les méthodes connues jusqu'à présent de classer les deux types de larves diploïdes selon leur sexe et d'étudier ensuite séparément les mâles diploïdes et les ouvrières. La nouvelle technique que nous décrivons ici pour diagnostiquer le sexe des larves d'abeilles fraîchement écloses repose sur les différences dans le contour et les dimensions de l'épiprocte (Fig. 1). Les larves mâles et femelles présentent des rapports de taille différents (Tab. I). Tout d'abord, chez le mâle une grande partie de l'épiprocte (un tiers à la moitié) est située au-dessus du pli présent dans la partie supérieure de l'épiprocte (Fig. 1A : a), chez l'ouvrière elle n'est d'environ que d'un quart (Fig. 1B : a). Ensuite, chez l'ouvrière seulement, la partie supérieure de l'épiprocte dépasse le niveau du contour dorsal du 9^{e} et avant-dernier segment abdominal (Fig. 1B), ce qui n'est pas le cas chez le mâle (Fig. 1A). Et enfin, la partie de l'épiprocte située sous le pli est chez le mâle (Fig. 1A : b) environ de la taille du sternum du 9^{e} segment (Fig. 1A : c), tandis qu'elle est nettement plus longue (Fig. 1B : c) chez l'ouvrière (Fig. 1B : b). La fiabilité de ce diagnostic du sexe chez les jeunes larves a été prouvée par différents tests. Par cette méthode on peut diagnostiquer le sexe très rapidement et, surtout, le faire sur des larves vivantes qui peuvent servir ensuite à d'autres recherches. Cette méthode permet aussi un élevage de mâles diploïdes en grandes séries.

***Apis mellifera* / diagnostic du sexe / épiprocte / mâle diploïde**

Zusammenfassung – Geschlechtsdiagnose bei frischgeschlüpften lebenden Larven der Honigbiene (*Apis mellifera)* erlaubt die Erkennung diploider Drohnen. Die Larven diploider Drohnen entwickeln sich bei der Honigbiene in Brutzellen desselben Typs wie die Arbeiterinnenlarven. Mit den bisher bekannten Methoden war es nicht möglich, die beiden diploiden

Larventypen nach ihrem Geschlecht zu sortieren und dann diploide Drohnen und Arbeiterinnen separat zu untersuchen. Die hier beschriebene neue Technik der Geschlechtsdiagnose von frischgeschlüpften lebenden Honigbienenlarven beruht auf Unterschieden in Konturen und Längenverhältnissen des Epiprokt (Abb. 1). Bei männlichen und weiblichen Larven liegen unterschiedliche Proportionen vor (Tab. I): Erstens wird bei Drohnenlarven ca. ein Drittel bis die Hälfte des Epiprokts abgefaltet (a in Abb. 1A), bei Arbeiterinnen dagegen nur ungefähr ein Viertel (a in Abb. 1B). Zweitens ragt das dorsale Ende des Epiprokts bei den Arbeiterinnen über das davor liegende 9. Abdominalsegment hinaus (Abb. 1B), was bei Drohnenlarven nicht der Fall ist (Abb. 1A). Und drittens ist der Teil des Epiprokts unter der Abfaltung bei Drohnenlarven (b in Abb. 1A) ungefähr so lang wie das Sternum des 9. Segments (c in Abb. 1A), bei Arbeiterinnen ist dieser Teil (b in Abb. 1B) dagegen deutlich länger als das Sternum (c in Abb. 1B). Die Zuverlässigkeit dieser Geschlechtsdiagnose bei Erstlarven wurde durch verschiedene Tests unter Beweis gestellt. Mit ihr kann eine Geschlechtserkennung sehr schnell und vor allem an lebenden Larven durchgeführt werden, die dann für weitere Untersuchungen zur Verfügung stehen. Sie erlaubt auch eine Aufzucht größerer Serien diploider Drohnen.

Geschlechtsdiagnose bei Erstlarven / Epiprokt / diploide Drohnen / *Apis mellifera*

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