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Original article

A new, simple method for rearing diploid drones in the honeybee (*Apis mellifera* L.)

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Abstract – Seven *Apis mellifera carnica* queens were instrumentally inseminated with the semen of their own sons. Diploid drone offspring of these queens were raised using two established techniques including elaborate laboratory manipulations, and a new approach. The new approach, based on routine beekeeping, uses small mating nuclei, which rear diploid drones to the adult stage late in the season. No labour and cost intensive feeding, nor grafting and incubation steps are needed. The ploidy level of the drone offspring was evaluated using seven DNA microsatellites. All drones reared by the elaborate techniques and more than 90% of the drones reared in a small mating nucleus were definitely diploid. This technique allows for easy and simple diploid drone rearing even in research groups with no sophisticated equipment.

***Apis mellifera* / honeybee / DNA microsatellite / diploid drone / instrumental insemination**

1. INTRODUCTION

Sex in honeybees (*Apis mellifera* L.) is determined at a single locus [1, 5]. Individuals that are heterozygous at the sex locus, develop into females, whereas individuals that are hemi- or homozygous develop into haploid or diploid males. Mother-son [11–13] or sister-brother [18, 19] inseminations are routinely used as a tool to obtain diploid drone offspring. The techniques developed by Woyke [19, 20] are well established. They involve the hatching of larvae in an incubator and, in several steps,

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grafting the larvae into artificial queen cells provided with royal jelly. Hence these techniques are expensive and labour intensive. In addition, they suffer from a rather low success rate and they are only applicable during the reproductive season, when colonies usually produce drones.

Recent observations of Polaczek [11] revealed that small mating nuclei headed by queens which have been inseminated with semen of their own sons may rear diploid drones to the adult stage very late in the season. There are three indications that diploid drones might be reared in these nuclei: (1) From routine bee keeping experience it is known that queens, which were not inseminated with semen of their own sons, do not lay drone eggs after the reproductive season. (2) The drones concerned hatched in worker cells. Normally, queens do not lay unfertilised eggs in worker cells [4]. (3) The sexual reproductive organs of these drones were compared with haploid drones [12, 13] following Woyke [21]. All comparisons revealed significantly smaller sexual reproductive organs of the drones raised in the small mating nuclei [10, 11] indicating that they were diploid.

In all cases, it might well be that young and/or insufficiently inseminated queens also lay haploid drones in worker cells. Therefore, a genetic control of the ploidy level of the drone offspring is necessary to prove the reliability of these techniques. The marker phenotypes *cordovan* and *chartreuse* have been used for reliability testing [17]. However, the maintaining of such highly inbred marker lines is very labour intensive. In contrast, DNA microsatellites [2, 3] allow fast reliability testing without interfering with routine bee breeding practice. Diploid drones can be identified via heterozygosity at discriminating loci. Here we present a new, simple diploid drone rearing technique based on the observations of Polaczek [11] and use DNA microsatellites for reliability testing.

2. MATERIALS AND METHODS

2.1. Experimental design

Seven virgin *Apis mellifera carnica* queens (A, B, C, D, E, F, G) were treated twice with CO_2 for 5 min to induce egg-laying of unfertilised eggs. Drone offspring were reared until the sexually mature stage. Then the queens were inseminated instrumentally using 8.14 ± 0.23 µl of mixed semen [6] of 12 of their own sons.

2.2. Diploid drone rearing techniques of Woyke

Potential diploid drone offspring of the queens A and B were raised according to established techniques [14, 18, 19] with the following modifications. Freshly hatched larval offspring of these queens were obtained from worker cells and were grafted into artificial queen cells. These queen cells were placed in a petri dish with water in an incubator at 34.5 °C. The larvae were repeatedly fed with a royal jelly/boiled water (1:1) mixture ad libidum. After 3 d the larvae were transferred into drone cells previously inhabited by drone larvae of the same age cohort and raised by a queenright mother colony until capping of the cells.

In order to confirm the approach of Woyke [18, 19] for rearing diploid drones in the colony, freshly hatched larval offspring (N = 6) of queen C from worker cells were grafted into artificial queen cells. Then, the larvae were reared for 12 d in a queenless mother colony until the offspring unambiguously revealed sex, i.e. drone offspring were still larvae, but females had already developed to pupae. After 3 d the open queen cells were turned horizontal to avoid the larvae from slipping out of the cells. Six other larvae of the same queen were treated following the approach used for queens A and B as a control. All Woyke techniques were performed during the months June and July, when European honeybee colonies usually rear many drones.

2.3. New method of rearing diploid drones

Based on the observations of Polaczek [10, 11] we further developed the following approach. Potential diploid drone offspring of the queens D, E & F, G were raised in small mating nuclei (c. 1000 workers) late in the season (end of September until end of October) and taken out daily for further investigations such as biotests. The nuke boxes (20 × 19 × 14 cm) were provided with two outer honey/pollen, two inner brood frames, a feeder (6 × 10 cm) and candy sugar (icing sugar/honey 3:1) ad libidum. Experiments were performed at the research apiary of the Free University of Berlin. Weather conditions were typical for autumn in Berlin, Germany indicating a three monthly mean [temperature: 9.0 °C ± 5.9 (1997), 8.1 °C ± 6.1 (1998); rain: 22.3 mm ± 2.1 (1997), 60.3 mm ± 4.1 (1998); sunshine: 120.3 hours ± 3.4 (1997), 71.9 hours ± 2.7 (1998); data from Meterologisches Institut, FU Berlin].

To investigate the number of diploid drones raised on a single day, the following approach was used. Two small mating nuclei (queens F and G) were each given one worker comb. After 48 h the combs were screened for eggs, and all eggs were marked on an overlaid transparency. After 5 d the combs were controlled for cannibalised brood using the transparencies. After 10 d the combs were screened for drone larvae.

2.4. DNA isolation and microsatellite/ genotype analysis

DNA isolation and genotyping was done from single drones (N = 12 each queen) following routine protocols [7–9, 15], and seven previously published DNA-microsatellites [2, 3]. Genotypes were analysed following routine procedures [3, 7–9]. Since each drone has a 50% probability of inheriting one or the other allele in case of queen heterozygosity at one locus, the non-detection error for heterozygosity of the queen at a tested locus is < 1% with 12 drones. In case a drone's ploidy level could not be unambiguously determined because of a lack of heterozygosity, we calculated the non-detection error. The chance of non-detecting diploid drones depends on the number of discriminating, heterozygous queen loci. With five loci this probability is smaller than 5%.

3. RESULTS

The experimental time, the number of grafted larvae, grafting experiments and diploid drones for the different diploid drone rearing techniques are presented in Table I. For the queens A & B we obtained a total of 120 diploid drones or 3.13% of the total number of grafted larvae. Queen C yielded 12 drones or 3.95% of the total number of grafted larvae, which were unambiguously diploid because of heterozygosity at least at one of the tested loci (the genotype data is available on request). No differences were found between the two modified Woyke approaches.

Our new approach yielded a total number of 160 diploid drones very late in the season (20 Sept.–3 Nov.). No drones were raised in the small mating nuclei in August and in early September. Among the drone offspring of queen D, raised in the small mating nucleus, two drones lacking heterozygosity at all loci were found. The non-detection error for one drone was smaller 5%.

For the nuclei F and G, the number of laid eggs, hatched and cannibalized larvae and reared diploid drones are presented in Table II. A total number of 397 eggs were laid after 24 h in the two mating nuclei. After 5 d 48.87% of the hatched larvae had not been cannibalized. After 10 d 2.06% of the cells were found to contain drones.

Table I. Number of diploid drones, grafted larvae and time for the different approaches of rearing diploid drones.

Method	Queens	Time	Grafting experiments	Larvae grafted after 3 d	Diploid drones	Diploid drones [%]
Woyke 1963	A	10.06. – 22.07.	19	2 811	85	3.02
	B	29.4. – 19.07.	14	1 027	35	3.41
	Total		33	3 838	120	3.13
modified	C (queen cells)	03.06. – 23.06.	5	131	6	4.58
Woyke 1963	C (drone cells)	03.06. – 23.06.	4	173	6	3.47
	Total		9	304	12	3.95
new approach	D, E	20.09. – 03.11.	–	–	57	
	F, G	19.09. – 01.11.	–	–	103	
	Total				160	

Table II. Number of diploid drones reared per day in the nuclei F and G.

Queen	Laid eggs (24 h)	Hatched larvae (5 d)		Eaten larvae (5 d)		Diploid drone larvae (10 d)	
	Number	Number	%	Number	%	Number	%
E	327	154	47.09	173	52.91	3	1.95
F	70	40	57.14	30	42.86	1	2.50
Total	397	194	48.87	203	51.13	4	2.06

4. DISCUSSION

We found clear genetic evidence that late in the season small honeybee nuclei rear diploid drones until the adult stage without any experimental manipulations. Moreover, the rearing of drones was possible at a time of the year when normal sized, mature colonies usually do not rear drones at all.

Our approach requires far less effort, because grafting steps and feeding of individual larvae are not involved. Moreover, the labour intensive raising of diploid drones in the incubator is not necessary. Additionally, the costs are distinctly lower, because feeding of larvae with royal jelly is not required. Finally, the success rate in terms of diploid drone offspring produced per day matches the other techniques. The number of diploid drones reared per day is nearly equal in both techniques [12, 13, 18, 19] after 10 d. Obviously, our approach does not work in summer because drones were not reared in the small mating nuclei. However, our approach enables the rearing of drones late in autumn, whereas the techniques of Woyke [18, 19] just work in summer, when colonies usually rear many male sexuals. Therefore, a combination of the different approaches would allow the rearing of diploid drones throughout the season. Diploid drone rearing in spring is generally difficult as a result of the low success rate of overwintering queens, which have been inseminated with the semen of their own sons [13].

The DNA microsatellite findings give strong support to the high reliability of the

two approaches of references [17, 18] to rearing references diploid drones in honeybees. Likewise, more than 90% of the drone offspring of queen D were definitely diploid, also indicating the high reliability of our technique.

Interestingly, the cannibalising of diploid drone offspring by workers seems to be dependent upon season and colony size. At the end of the season diploid drones were reared up to the adult stage in the tested nuclei. This is different to normal sized colonies, where diploid drones are never reared until hatching. Since diploid drones were not reared in the small mating nuclei in summer, we argue that the combination of colony size and season is highly important for our findings. This may indicate that either the behavioural threshold of workers for cannibalising diploid drone offspring is reduced or that workers execute their ability to recognise diploid drones only during the reproductive season.

We conclude that our approach is a simple, fast and easy technique for rearing diploid drones without using any sophisticated equipment. The chance of sampling haploid drones is small. If however, accuracy about the ploidy level is needed, the reliability remains to be tested for each drone using appropriate genetic methods as shown in this report.

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Résumé – Méthode nouvelle et simple pour élever des mâles diploïdes chez l'abeille domestique, *Apis mellifera* L. Sept reines d'*Apis mellifera carnica* (A à G) ont été inséminées avec le sperme de leurs propres fils. À partir des reines A, B et C ont été élevés des mâles diploïdes selon les méthodes connues [14, 18, 19]. Les autres reines (D à G) ont été placées dans des cagettes de fécondation et des mâles diploïdes ont été prélevés chaque jour tard dans la saison. Le tableau I donne le nombre de mâles diploïdes, de larves greffées et la durée des différents modes d'élevage. Le tableau II donne le nombre de mâles diploïdes élevés en une journée dans une cagette d'élevage. Le génotype de 12 mâles issus des reines C et de 12 mâles issus des reines D a été déterminé à l'aide de sept microsatellites d'ADN. Tous les mâles de la reine C et 90 % de ceux de la reine D étaient hétérozygotes à au moins l'un des loci et donc diploïdes. Notre technique permet, avec une grande fiabilité, d'élever de façon simple et peu coûteuse des mâles diploïdes sans manipulation expérimentale. Notre nouvelle approche combinée aux méthodes existantes permet l'élevage de mâles diploïdes durant toute la saison apicole.

***Apis mellifera* / mâle diploïde / insémination artificielle / microsatellite ADN**

Zusammenfassung – Eine neue, einfache Methode zur Aufzucht diploider Drohnen der Honigbiene (*Apis mellifera* L.). Sieben *Apis mellifera carnica* Königinnen [A, B, C, D, E, F, G] wurden mit Samen von ihren eigenen Söhne begattet. Von den Königinnen A, B, C wurden diploide Drohnen nach etablierten Methoden aufgezogen [14, 18, 19]. Die anderen Königinnen [D, E, F, G] wurden in kleine Begattungskästchen eingesetzt, und spät in der Saison wurden täglich diploide Drohnen entnommen. Die Anzahl der diploiden Drohnen, umgesetzten Larven und der Zeiträume für die verschiedenen Ansätze sind Tabelle I zu entnehmen. Tabelle II zeigt die Anzahl diploider Drohnen, die an einem Tag in einem Zuchtkästchen aufgezogen werden. Je 12 aufgezogene Drohnen der Königinnen C & D wurden mit sieben DNA-Mikrosatelliten genotypisiert. Alle Drohnen der Königin C und mehr als 90 % der Drohnen von Königin D waren mit Sicherheit diploid,

aufgrund von Heterozygotie an mindestens einem der Loci. Unsere Technik erlaubt daher mit hoher Sicherheit eine einfache und kostengünstige Aufzucht diploider Drohnen ohne jegliche experimentelle Manipulation. Eine Kombination von unserem neuen Ansatz mit etablierten Methoden erlaubt eine Aufzucht diploider Drohnen über die ganze Saison.

***Apis mellifera* / diploider Drohne / instrumentelle Besamung / DNA-Mikrosatellite**

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