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THE PRESENCE OF SPERMATOZOA IN EGGS AS PROOF THAT DRONES CAN DEVELOP FROM INSEMINATED EGGS OF THE HONEYBEE*

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SUMMARY

A search for spermatozoa was made in 411 eggs 1 – 3 hours old, taken from worker and drone cells. The eggs originated from one naturally mated queen and six sibling-mated queens that laid eggs in worker cells of which 50% were male (drone).

Spermatozoa were found in only one out of the 62 eggs taken from drone cells, in those from worker cells the number of spermatozoa ranged from 1 to 8, in proportions 60 – 65%, 20%, 10%, 2%, 1%, 0·5%, 0·0%, 0·5%. A low percentage of eggs containing only 1 – 2 spermatozoa was correlated with a high maximum number of spermatozoa in other eggs of the same queen. No relation was found between the number of spermatozoa in the spermatheca (deduced from the amount of semen) and the number in eggs laid by the same queen.

Spermatozoa were found in 93·2% of the 295 eggs laid by the sibling-mated queens in worker cells and in 88·9% of the 54 laid by the naturally mated queen. It is concluded, that male larvae in worker cells from eggs laid by the sibling-mated queens must have hatched from eggs into which the spermatozoa had penetrated.

INTRODUCTION

After the hypothesis of development of drone honeybees (*Apis mellifera*) from unfertilized eggs was put forward by Dzierzon (1845), the microscopical examinations of honeybee eggs by Siebold (1856), Blochman (1889), Paulcke (1899), Petrunkewitsch (1901) and Nachtsheim (1913) showed the presence of spermatozoa in eggs taken from (female) worker cells. On the other hand these authors found no spermatozoa in eggs from (male) drone cells. Together with other work, this formed the basis of the theory of the development of drones from unfertilized eggs. The egg may be unfertilized because it is uninseminated, or because (although inseminated) the sperm pronucleus did not combine with the egg nucleus.

After Whiting's explanation of sex determination in the parasitic wasp *Habrobracon* (1943), one aspect of sex determination in the honeybee again became prominent: could a fertilized egg ever produce a drone? Mackensen (1951) showed that in matings of closely related queens and drones, many eggs failed to produce adults (as in *Habrobracon*), but (in contrast to *Habrobracon*) no diploid drones—resulting from fertilization—were found. A few years later Rothenbuhler (1957), and Drescher and Rothenbuhler (1964), obtained genetic evidence that certain patches in compound eyes of genetically mosaic drones were

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composed of diploid male tissue resulting from fertilization. Meanwhile the present author (Woyke, 1963*a*, *b*) had demonstrated that (after sibling mating) certain queens lay eggs in worker cells that give rise to female and male larvae in the ratio 1 : 1. Since eggs laid in worker cells are normally inseminated, the

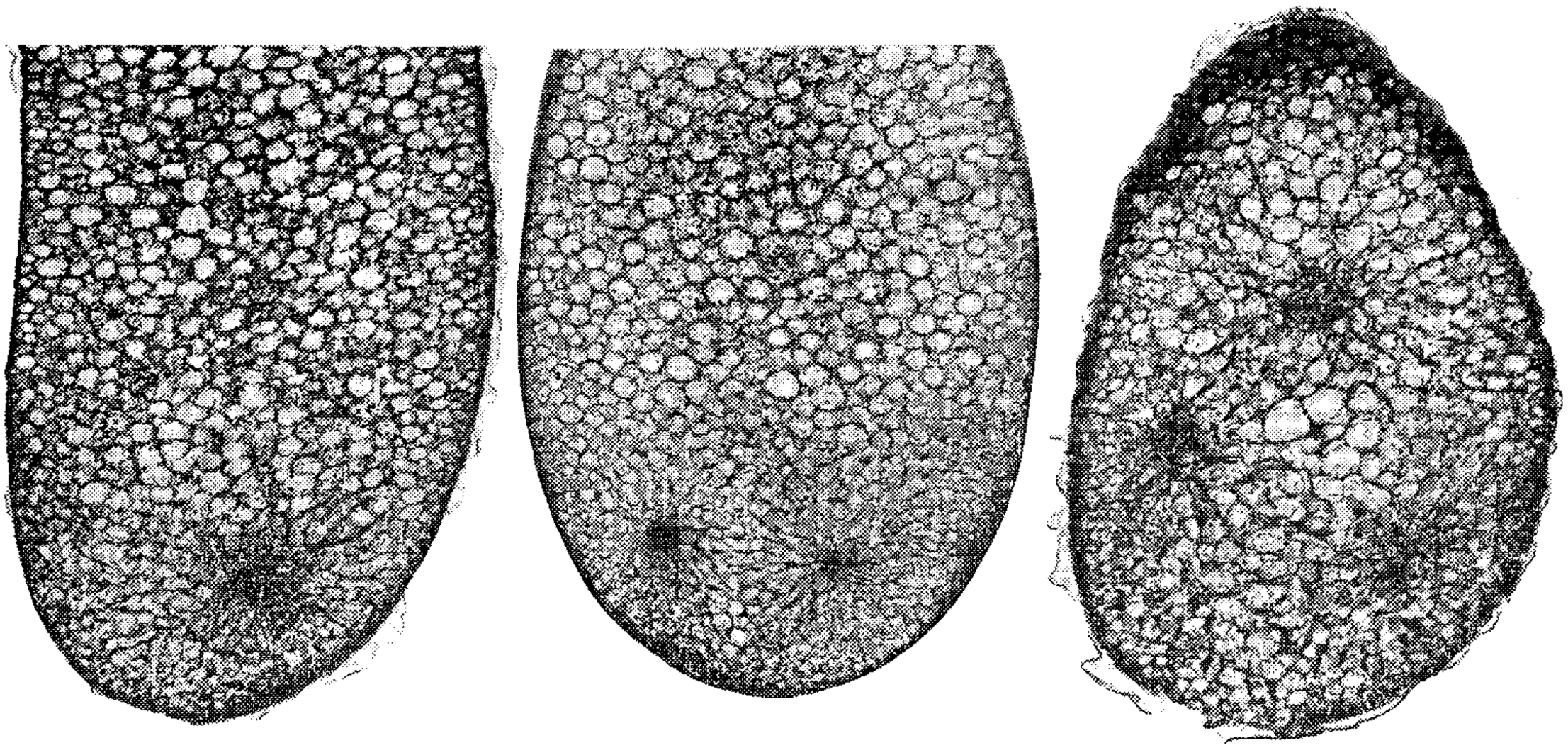

FIG. 1. Eggs 1 – 3 hours old showing radiation area around (*a*) 1 spermatozoon, (*b*) and (*c*) 2 and 3 spermatozoa

drones were presumed to have developed from inseminated eggs. Cytological evidence was, however, lacking, and the present investigations were made in order to obtain this. A preliminary report has been published (Woyke, Knytel & Bergandy, 1965).

MATERIAL AND METHODS

Ten queens were used; one was mated naturally and the others instrumentally, using sibling drones. About 500 eggs were investigated; of these 90 were used to establish the age of the egg at which a spermatozoon is distinctly identifiable in it. Next, to solve the main problem, a search for spermatozoa was made in 411 eggs (from the naturally mated queen and from six of the sibling-mated queens, which produced brood in worker cells showing 50% survival). Of these eggs 62 were collected from drone cells and 349 from worker cells; among the 349 were 54 laid by the naturally mated queen.

The general plan of this investigation was to establish the survival rate of brood produced by the sibling-mated queens, to determine the sex of larvae, pupae and imagines from worker cells, and next to search for spermatozoa in eggs collected from drone and worker cells. Table 1 shows that with only two queens (854, 885) was it possible to follow all the above steps. The frequent opening of hives led to loss of queens, and this forced us to carry out some of the steps on different queens.

Queens which were themselves the first sibling-mated generation were inseminated instrumentally with semen of one drone only: those originating from

a mother already producing brood of 50% survival rate were given more semen (Table 1). Next, by the method described earlier (Woyke, 1963*a*), a check was made for queens only 50% of whose brood survived in the colony.

TABLE 1. Details of sibling-mated queens used

First generation of sibling-mated				*Second generation of sibling-mated*			
Queen no.	*Volume of semen (cu. mm.)*	*Survival rate of brood*	*Brood investigated*	*Queen no.*	*Volume of semen (cu. mm.)*	*Survival rate of brood*	*Brood investigated*
751	1·2	49%	eggs W	854	6·0	54%	older larvae and imagines (sexed), eggs D, W
754	1·2	69%	larvae 1 day old (sexed)	882	2·5	52%	eggs W
763	1·0	22%	larvae 1 day old (sexed)	883	2·5	42%	larvae 1 day old and older pupae (sexed)
770	1·2	43%	larvae 1 day old (sexed), eggs D	885	2·5	43%	older larvae, pupae, imagines (sexed), eggs D, W
881	0·9	49%	older larvae (sexed), eggs W				

D=eggs from drone cells W=eggs from worker cells

To be certain that the eggs laid by these queens in worker cells gave rise to drones, larvae from eggs of different queens were hatched and reared in an incubator, by methods already described (Woyke, 1963*c*). The young larvae were sexed by histological (Woyke, 1963*a*) and anatomical (unpublished) methods. Next, diploid drones were reared to the imago (preliminary report Woyke, 1965).

To establish the best age of eggs for confirming the presence of spermatozoa, eggs 0 – 2, 1 – 3 and 2 – 4 hours old (collected by the method in the next paragraph) were examined. For greater accuracy an observation hive was also used. The laying queen was watched and the place and time of deposition of each egg was noted. The eggs, whose age was exactly known, were also examined in sections on slides.

The main investigation of this paper was carried out on eggs 1 – 3 hours old. They were collected only from those sibling-mated queens producing 50%-survival brood (the other 50% being drone larvae, eaten by the workers), and for comparison also from the naturally mated queen. With this in view, the queens were caged under a queen-excluder cover on worker or drone comb for two hours, and the comb was then placed for one hour in an incubator. Thereafter the eggs were fixed with Petrunkewitsch's fluid, sections were stained with Heidenhein's iron haematoxilin and counter-stained with eosin, and the eggs were then examined for spermatozoa.

RESULTS

Verification of queens

From the test for survival rate, it proved (Table 1) that all four of the sibling-mated queens which originated from a mother already producing brood of 50% survival rate also produced such brood. Of the five queens constituting the first generation of sibling-mated, three produced brood of 50% survival rate, one (754) produced brood of a high (69%) and the other (763) of extremely low (22%) survival rate.

Sexing of 100 larvae hatched in worker cells in an incubator and kept there for one day showed that 50% of larvae from queens 770 and 883 were drones. Larvae from the other two sibling-mated queens (754, 763) produced brood whose survival rate was very different from 50%, only occasional drone larvae being found. So of these queens, only 770 and 883 were used for further investigation.

Rearing larvae hatched in worker cells from eggs of queens 854, 881, 883 and 885 gave rise to the following numbers of worker and drone larvae at the time of sealing: 26 and 32, 16 and 11, 46 and 56, 92 and 168, a total of 180 females and 267 males. The sex ratio at sealing was not the same as that for eggs or hatched larvae, because of the rearing conditions. Many adult drones were also reared from brood of such queens; detailed results of rearing the 'diploid' drones to maturity will be published later.

Age of eggs in which the presence of spermatozoa can be established with certainty

The spermatozoon is difficult to identify immediately after it has penetrated into the egg. Soon, however, the cell plasma forms a radial structure round the head, thereby showing the presence of the spermatozoon. The radial structure can already been seen well in eggs 1 hour old. The union of male and female pro-nuclei can be seen in eggs 4 hours old. Eggs 1 – 3 hours old were therefore chosen for the main study.

Investigation of eggs collected from drone cells

Only one egg of all those laid in drone cells by three sibling-mated queens was found to contain a spermatozoon (Table 2). This single exception may be due to the fact that the queen was artificially forced to lay eggs in a drone comb by being caged under a cover, in which circumstances it can happen that she lays

TABLE 2. Results of examination of eggs 1 – 3 hours old laid in drone cells by sibling-mated queens

Queen no.	*No. eggs examined*	*No. without spermatozoa*	*No. with spermatozoa*
770	33	33	0
854	9	9	0
885	20	19	1
Total	62	61	1
Percentage	100%	98·4%	1·6%

inseminated eggs in all the drone cells. But in the present study this was not so, and practically all the eggs laid in drone cells (98·4%) were uninseminated.

Investigation of eggs from worker cells

A single spermatozoon was found in 38·8 – 77·2% of eggs laid in worker cells by sibling-mated queens (Table 3), so some queens laid twice as many eggs with one spermatozoon as other queens did. Altogether 59·0% of the eggs of sibling-mated queens contained one spermatozoon; for the naturally mated queen the ratio was 68·5%. Since this is within the range for sibling-mated queens, eggs of the two types of queen do not seem to differ significantly in this respect.

Two spermatozoa were found in 6·3% – 37·1% of the eggs from individual queens. The variation is even higher than that shown previously: one queen (751) laid ten times as many eggs with one spermatozoon as with two, whereas another (885) gave about equal numbers of each type.

Three spermatozoa were found in eggs of each queen (Table 3), but the average proportion of such eggs was 2·5 – 16·0%. More than three (even up to eight) spermatozoa were found only in eggs of certain queens, the percentage of such eggs being 1·7 – 5·3%. More than three spermatozoa were found in the eggs of queens which produced a relatively low percentage of eggs containing only one spermatozoon. Thus a low percentage of eggs with only one spermatozoon is connected not only with a higher percentage of eggs containing few spermatozoa but also with a high number of spermatozoa in occasional eggs of the same queen.

TABLE 3. Spermatozoa in eggs 1 – 3 hours old collected from worker cells in colonies where the sibling-mated queen produced 50% drone larvae

Sibling-mated queen no.	*No. eggs examined*	*Percentage of eggs with the following no. spermatozoa* 1	2	3	4	5	6	7	8	*Total % eggs sperms found*	*sperms not found*
751	79	77·2	6.3	2·5	0	0	0	0	0	86·0	14·0
854	57	57·9	22·8	12·3	5·3	1·7	0	0	0	100·0	0
881	50	48·0	20·0	16·0	0	2·0	2·0	0	2·0	90·0	10·0
882	55	63·7	20·0	12·7	0	0	0	0	0	96·4	3·6
885	54	38·8	37·1	14·8	3·8	1·8	0	0	0	96·3	3·7
Total	295	59·0	20·0	10.8	1·8	1·0	0·3	0	0·3	93·2	6·8
Queen mated naturally	54	68·5	13·0	3·7	3·7	0	0	0	0	88·9	11·1
Total for all queens	349	60·4	19·0	9·7	2·0	0·8	0·3	0	0·3	92·5	7·5

Although this tendency is clear, data presented in Table 3 show that eight spermatozoa (the highest number found) were not found in eggs of queen 885, which produced the lowest percentage of eggs with one spermatozoon; she did, however, lay many eggs containing two.

An attempt was made to compare the output of queens whose eggs contained low and high numbers of spermatozoa. Numbers of eggs containing 1 or 2 spermatozoa were added together; as the percentage (of the total number this represents) *decreases* from 83·7% to 68% (Table 3, columns 3+4), the maximum number of spermatozoa in other eggs of the same queens *increases* from 3 to 8. The coefficient of this correlation is very high; $r = 0{\cdot}97$.

If not all eggs are taken into account, but only those containing spermatozoa, the data in Table 4 are obtained. As the maximum number of spermatozoa found in *any* egg of a particular queen increases from 3 to 8, there is an increase from 2·9% to 24·4% in the percentage of eggs containing more than two spermatozoa ($r = 0{\cdot}82$), and from 1·13 to 1·93 in the average number of spermatozoa per egg. From the rather small amount of data so far available, it would seem that queens producing relatively more eggs with many spermatozoa also lay eggs with highest maximum number. The result may have a general application, since the data for each queen represent the sum from several samples collected over a period of time. Nachtsheim (1913) also rarely found less than 3 spermatozoa per egg (more than we did), and he found also higher maximum number (10 per egg).

TABLE 4. Some relations between the numbers of spermatozoa found in eggs laid by individual queens

Queen no.	*No. eggs containing spermatozoa*	*% with more than 2*	*Max. no. found in 1 egg*	*Av. no. per egg*	*Semen used for insem. queens (cu. mm.)*
751	68	2·9	3	1·13	1·2
nat.	48	8·4	4	1·35	11·0?
882	53	13·2	3	1·47	2·5
854	57	19·3	5	1·70	6·0
885	52	21·1	5	1·88	2·5
881	45	24·4	8	1·93	0·9

Table 3 and 4 show that eggs with higher numbers of spermatozoa were not laid more frequently by the naturally mated queen than by those mated instrumentally with a small volume of semen. Among these, no correlation was found between the volume of semen used for artificial insemination and the number of spermatozoa in eggs (Table 4). Thus it seems that the amount of semen received by the queen, and consequently the number of spermatozoa in her spermatheca (Woyke, 1960; Mackensen, 1964) does not influence the number of spermatozoa in the eggs laid. The number of spermatozoa in eggs may depend upon the characters of spermatozoa, but it certainly seems to depend on characteristics of the eggs laid by individual queens.

The results for eggs laid by the naturally and instrumentally mated queens do not differ appreciably, so it seems possible to draw general conclusions from the total numbers for all the queens.

Numbers of spermatozoa 1, 2 and 3 were found roughly in 60% (or 65% excluding eggs with no spermatozoa), 20% and 10% of eggs respectively, 4 and 5

spermatozoa in about 2% and 1%, and a greater number in less than 0·5% of all eggs investigated.

Table 3 shows that 86 – 100% of all eggs of each queen were found to contain spermatozoa. Spermatozoa were absent in some eggs originating from the instrumentally sibling-mated queens, as well as in some from the naturally mated queens. The percentage of eggs in which the spermatozoa were found was lower for the naturally mated queen than for any other. Altogether spermatozoa were found in 93·2% of eggs laid by the sibling-mated queens and in 88·9% of those from the naturally mated one. Thus the absence of spermatozoa in 6·8% of eggs from the sibling-mated queens was caused neither by instrumental insemination with small amount of semen, nor by inbreeding.

Spermatozoa might be absent in some eggs of each queen because these eggs were not inseminated. But in this case drone (haploid) brood sealed with the domed capings should be present in the worker combs of the colony, and this was not so. Absence of spermatozoa in some eggs is to be explained rather by technical difficulties, such as investigation of the eggs at too early a stage of development, when it is difficult to find the spermatozoa, or destruction and loss of some section during the preparation of slides.

The high percentage of eggs in which the spermatozoa were found, as well as the fact that this percentage was not lower (but even higher) for sibling-mated queens than for others, shows that these queens laid in worker cells eggs similar to those laid by naturally mated queens, i.e. inseminated.

CONCLUSIONS

If all the eggs of sibling-mated queens, or only the 93·2% in which the spermatozoa were found, are regarded as inseminated, the percentage is about twice as high as the 50% ratio of eggs from which females (workers) develop. Drone larvae must thus develop from the rest, i.e. from the 43·2 – 50·0% of the inseminated eggs. Since the sibling-mated queens produce drone larvae only from about 50% of eggs laid in worker cells, practically all the drone larvae must have developed from inseminated eggs.

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