DRONE LARVAE FROM FERTILIZED EGGS OF THE HONEYBEE*

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SUMMARY

Queens bred for two or three generations by brother-sister matings laid fertilized eggs in worker cells, from which larvae hatched, but only 50% of them survived in the hive.

Pieces of worker combs containing eggs laid by these queens were put into an incubator, and the hatched larvae grafted into queen cells on royal jelly and left in the incubator for 24 hours. The sex of these larvae was determined by microscopic anatomical investigations; it could be shown that about 50% of these larvae were male and 50% female. Thus males can develop not only from unfertilized, but also from fertilized eggs; such diploid drones are not seen as adults in the hive, because the diploid drone larvae disappear from the cells within a few hours of being hatched.

A new mechanism of sex determination in the honeybee is suggested on the basis of these results: a series of alleles exists at locus X; heterozygotes result in females, but azygotes and homozygotes in males. It is therefore proposed that locus X be called the sex-determining locus, and the alleles at this locus, sex alleles.

INTRODUCTION

Dzierzon (1845) established that drone honeybees develop from unfertilized eggs, but queen and workers from fertilized ones. This mechanism of sex determination in the honeybee has so far been generally accepted. But there are some Hymenoptera, in particular *Habrobracon*, in which diploid as well as haploid males can be found (Whiting, 1940, 1943; Speicher & Speicher, 1940). The diploid males are homozygous at locus X; many of the homozygous eggs do not hatch, and the diploid males have a low viability.

Cases were observed many years ago in which adults did not develop from all the eggs laid by a queen honeybee. Normally, inseminated queens lay unfertilized eggs in drone cells, from which drones develop, and fertilized eggs in the smaller worker cells, from which workers develop. Brood of similar age is present in the adjacent cells, which makes a 'compact brood nest'. But it sometimes happens that the brood disappears from some of the worker cells, and the queen then lays new eggs in these empty cells, giving the brood a 'scattered' appearance. It has been believed that the disappearance of brood is due to lethal eggs homozygous at locus X. Hadorn (1961) has discussed the general problems of lethals in insects, including the mechanism of dying.

Homozygosity can be obtained by inbreeding. Mackensen (1951) and Hachinohe and Jimbu (1958) were able to demonstrate that after sibling mating some honeybee queens produced scattered brood. Then Mackensen (1955), and Laidlaw, Gomes and Kerr (1956), demonstrated that this also sometimes follows mating between unrelated parents. It was concluded that the mates might have one allele at locus X in common. Eleven different alleles were shown by Mackensen (1955) to exist in six inbred lines; Laidlaw, Gomes and Kerr (1956) estimated that there were $12{\cdot}4 \pm 3{\cdot}56$ such alleles in one random-mating population of 65 colonies of honeybees in Brazil. Daily counts of

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brood from these queens showed that the greatest loss occurred between the third and fourth days, or at the time of hatching. It was concluded that the homozygous eggs are lethal, do not hatch, and are removed by the bees. The existence of a haplo-viable but homozygous lethal series of alleles in honeybees was thus assumed.

Rothenbuhler (1957) obtained mosaic drones in which some patches in eyes were probably diploid; he suggested that this tissue was able to survive only by virtue of its association with normally viable haploid male tissue. But Woyke (1962) showed that eggs homozygous at locus X are viable and hatch, and that the very young *larvae* disappear from the cells. There has so far been no detailed investigation of the disappearing brood, and we therefore attempted to determine its sex.

METHODS

Homozygosity is approached by inbreeding. Kalmus and Smith (1948) have worked out the results of various mating systems, including brother-sister mating used in the present experiments. This results in half the eggs being heterozygous at locus X, and half homozygous.

Queens were mated to their brothers for two or three generations, each queen with one drone only. After the first mating, all generations were bred from queens producing brood of low survival rate. After insemination, the queens were prevented from leaving the hive until they had started to lay eggs. The drones emerged in isolaters and were maintained in them, or in special cages. Drones ejaculating much semen (mostly 1·4 cu. mm.) were chosen for insemination. Queens which, due to lack of semen, laid unfertilized eggs in worker cells were eliminated.

Data presented in this paper concern the brood of four queens, no. 266 being the mother of the other three. Survival rate was tested as follows. After the queen (free or under a queen-excluder cover) had laid eggs in a comb for one day, she was recaged on another comb; cardboard with a rectangular hole was placed over the first comb, and normally deposited eggs within the area counted. Counts were repeated on the fifth day, when the youngest larvae were 1 day old.

Because the eggs in worker cells are fertilized, their sex can only be determined anatomically, and only at the last stage of embryonic development. Pieces of worker combs containing eggs 3 days old, from queens producing low-survival brood, were placed in incubators at 34·5°C., in a desiccator or a Petri dish with a little water in the bottom. Before they hatched, the eggs were fixed in Petrunkiewitsch or Tellyesniczky fluid, then the embryos were removed from the chorion and embedded normally in paraffin. About 400 embryos were prepared in this way.

In other experiments the pieces of comb in an incubator were checked every 3 hours, and hatched larvae were fixed; in the most recent experiments they were grafted on royal jelly into queen cells, 4 to 8 larvae per cell. The cells were put in an incubator, in a desiccator containing a little water. At first the larvae were left on the royal jelly for 6–9 hours, later for about 24 hours. They were fixed in Tellyesniczky fixative, and stained with Congo red before embedding in paraffin. This made it possible to determine the sex of the larvae in the paraffin sections even before dissolving the paraffin. Some sections were stained in Delafield or Heidenhain haematoxylin and eosin, and mounted in Canada balsam. For comparative purposes sections were made of larvae taken from worker and drone cells in a colony headed by a naturally mated queen producing normal brood.

RESULTS

Test for survival rate

Table 1 shows that only about 50% of larvae survived from eggs laid by queen 266, indicating that in this colony the number of alleles at locus X was reduced to two. The other three queens (and the drones with which they were mated) originated from queen 266, so it was expected that all would produce brood of 50% survival rate. This was confirmed visually with queen 580, and by counts for 581 and 652.

TABLE 1. Survival rate of brood produced by queens bred by individual sibling mating

Queen no.	*No. eggs laid*	*No. larvae 5 days after eggs laid*	*Percentage of larvae surviving*	*Percentage of larvae disappearing*
266	280	136	48·6	51·4
580	not counted	scattered	about 50	about 50
581	213	112	52·3	47·7
652	196	108	55·1	44·9

Investigations on embryos

According to Zander, Löschel and Meier (1916) sex can be determined from sections of larvae 3–6 hours old. It was therefore thought that this might also be possible with embryos a few hours before hatching, but difficulties were encountered. In younger embryos we could not with certainty determine the sex anatomically. Many older embryos, however, shrank during dehydration and embedding, and it was difficult to determine the sex ratio accurately. Since at that time the author believed that, as stated in the literature, eggs homozygous at locus X do not hatch, the problem seemed hopeless, but the discovery that low survival brood can hatch (Woyke, 1962) provided an alternative method.

Rearing of larvae and their viability

Sections of larvae just hatched in dry cells in the incubator were not much better than those of older embryos, but when the larvae were grafted on to royal jelly, so that the alimentary canal became filled, very good sections were obtained.

Because most of the larvae disappeared *from the hive* in 6–9 hours after hatching, the larvae were also left in the incubator for 6–9 hours. But to our astonishment almost all these larvae were alive after that time; it was possible to keep them alive for 24 hours and even more; detailed data on the viability of these larvae will be published in another paper. Data collected for that investigation and the present one, concerning altogether 656 larvae, show that 93·6% of the grafted larvae were alive after 24 hours. But about half these larvae would undoubtedly have disappeared if left in the hive.

Sex of the larvae

Examination of sections of the larvae reared in the incubator showed them to be of two types. Those of the first type (Fig. 1*a*) had smaller rudiments of gonads *G*; the two mesodermal cords extending from these gonads ended on the ventral side of the tenth segment, and three pairs of imaginal discs *ID* were found on the ventral side of the abdomen, on segments 10, 11 and 12. Larvae of the second type (Fig. 1*b*) had larger rudiments of gonads *G*; the two mesodermal cords *MC* extending from the gonads ended on the ventral side of segment 12, and only one imaginal disc *ID* was found on the ventral side of the abdomen, namely on segment 12. A depression ran from the middle of the disc towards the enlarged endings of the two mesodermal cords. It was thus clear from Zander, Löschel and Meier's investigations, and our comparative studies, that the larvae of the first type (Fig. 1*a*) were females and those of the second (Fig. 1*b*) were males.

Sections through the abdomen of larvae hatched in worker cells from fertilized eggs laid by a queen producing brood of low survival rate.

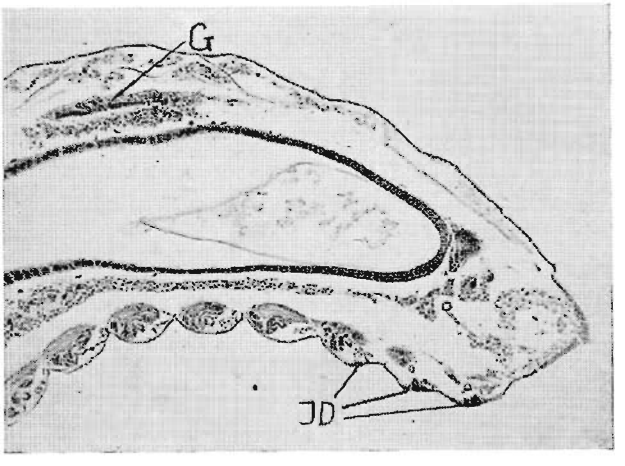

Fig. 1*a*. Female larva

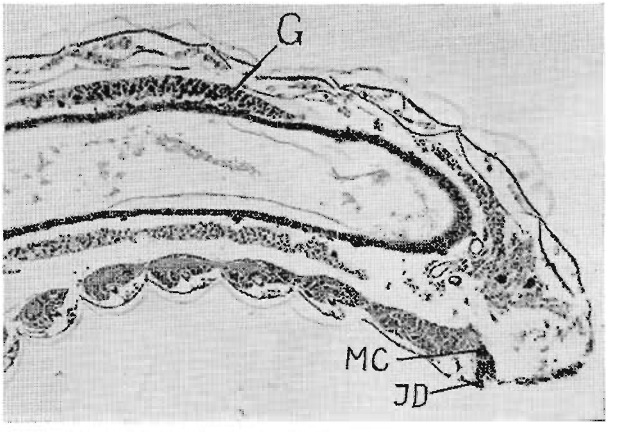

Fig. 1*b*. Male larva

G rudiment of gonad (ovary in female, testis in male)
ID imaginal discs
MC end of mesodermal cord extending from gonad to imaginal disc (rudiment of seminal duct and mucus gland)

It was very easy to determine the sex of larvae 24 hours old. It was also possible to determine the sex of some of the younger larvae that had died in the incubator, from similar sections.

The summarized results in Table 2 show that about 50% of larvae were drones and the other 50% workers. Thus both females and males can develop from fertilized eggs of the honeybee.

TABLE 2. Sex of larvae hatched in the incubator from eggs laid in worker cells by queens producing brood of low survival rate

Queen no.	*No. larvae investigated*	*Percentage female*	*Percentage male*
266	81	49·4	50·6
580	19	42·1	57·9
581	68	50·0	50·0
652	110	53·6	46·4
Total	278	50·7	49·3

Which larvae disappear ?

Comparison of Tables 1 and 2 shows considerable agreement between the percentages of brood surviving and of female to male larvae. Of the brood examined in detail, that of queen no. 266 showed the lowest survival rate, and also included the lowest percentage of females. On the other hand, queen 652 produced brood of which the highest percentage survived, and which contained the highest percentage of females.

The agreement between the percentage of disappearing brood and the percentage of males produced in worker cells by the same queens shows that it is practically only the drone larvae which disappear from workers cells in the hive. A further paper will describe what happens to the larvae that disappear in the hive. On the basis of different types of mating it is known that larvae heterozygous at locus X survive, and it can therefore be assumed that drone larvae from fertilized eggs, and which disappear from the cells, are homozygous at locus X.

CONCLUSIONS

On the basis of the investigations described above, a new mechanism of sex determination should be accepted for the honeybee, in addition to male haploidy. It has been demonstrated that both females and males can develop from fertilized eggs. This result, together with those of earlier investigations on ' lethal alleles ', shows that a series of alleles exists in the honeybee at locus X, heterozygous combinations *ax/xb* of which result in females, azygotes *xa* or *xb* and homozygotes *xa/xa* in males. Drone larvae are thus of two types; those from azygotes are haploid in origin, and are impaternate, and those from homozygotes are diploid and have two parents. Adult diploid drones are not seen in the colony, because diploid drone larvae disappear from the cells soon after hatching. In conformity with suggestions (Rothenbuhler, 1958) on abbreviated names of genes and mutations, it is proposed to call locus X the sex-determining locus, and the alleles at this locus the sex alleles.

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