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REARING AND VIABILITY OF DIPLOID DRONE LARVAE*

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SUMMARY

Four queens, giving brood only half of which normally survived, were bred by individual sibling mating. To protect the brood of these queens from bees, pieces of combs containing eggs were put into the incubator, without bees. After the eggs hatched, 707 larvae were grafted into queen cells on royal jelly, and were reared further in the incubator. On the fifth day the sex of the live larvae could be determined: half were female and half male. The viability of the diploid drone larvae was no lower up to this time than that of female larvae. Larvae were reared further, giving both queen and drone pupae and imagines. The larvae from so-called 'lethal eggs', which are normally eaten in the hive, are thus viable diploid drones from fertilized eggs. This paper also reports for the first time the rearing of adult drones and of normal queens, beginning from the egg stage, outside the hive without any contact with bees.

INTRODUCTION

Work on larvae from 'lethal' eggs of inbred honeybee queens (*Apis mellifera*) has been continued. It was shown (1962) that of the eggs laid in worker cells by sibling-mated queens producing brood of low survival rate, all hatch, but 50% of the larvae disappear from the cells within a few hours of hatching. Histological examination (1963*a*) showed that the disappearing larvae were drones. In the colony they are eaten alive by the worker bees (1963*b*). The eggs were not 'lethal', nor were the larvae.

The present investigation concerns the viability of the drone larvae hatched from these fertilized eggs, and our attempts to keep the diploid drone larvae alive to the pupal and imaginal stage.

MATERIAL AND METHODS

Queens producing drone larvae from fertilized eggs were obtained by individual sibling mating for two or three generations. The survival rate of their brood was studied by methods already described (Woyke, 1962, 1963).

Four queens producing brood of 50% survival were chosen. The viability of the 'disappearing' drone larvae was investigated as follows. To protect the drone larvae from being eaten by the workers, all eggs were hatched, and all larvae reared, without bees, in the incubator. The other 50% of the brood (female and normally not eaten by the workers) served as a control group.

Altogether 707 larvae hatching in the incubator were reared in the following way. A comb with worker cells was put in a hive headed by one of the four queens, and inspected every day for eggs. Sometimes the queen was covered on the comb with a queen-excluder cage for a day. Before her first eggs were three days old, pieces of comb containing these eggs were cut out, and put in the incubator after the cell walls had been cut down somewhat. (If eggs of an unknown age had to be used, they were in cells near larvae which

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had started to hatch.) The pieces of comb were placed in a moistened Petri dish or in a desiccator with a little water on the bottom. The eggs were inspected every three hours.

All hatched larvae were grafted on to royal jelly, in a queen cell from which a queen larva had been removed, 4–10 larvae per queen cell. Every 24 hours they were transferred on to fresh royal jelly. The number of larvae in each queen cell was gradually decreased on subsequent days. On the third or fourth day larvae were usually grafted on to a glass dish supplied with food, a few to each dish.

Initially not much attention was devoted to the age of the queen larvae removed, as long as this was not more than three days. Later, the royal jelly used was always from queen larvae of the same age as the larvae grafted on it. Sometimes the larvae hatching in the incubator were first grafted on bee milk from worker cells in the hive, from which larvae just hatched had been removed. In the final experiments, larvae four days old were transferred on mixed food taken from cells of older worker larvae, instead of on royal jelly.

All the grafted larvae were kept in the incubator in a desiccator fitted with a water trap. The queen cells with young larvae were suspended (as in the hive) or held upright. Queen cells with older larvae were always kept upright. The relative humidity was maintained by a sulphuric acid bath at 96% (acid s.g. 1·05) in early experiments; later it was maintained at 100% during the first day of larval life, and then decreased to 96%.

Female larvae were transferred for pupation on the 6th-7th day, drone larvae on the 9th–10th day. The time of transference depended on the size of the larvae, commencement of spinning, and voiding of the transparent yellowish liquid. Larvae fed all the time on royal jelly were transferred to queen cells just sealed, an opening having been cut in the side wall and the queen larva removed. The queen cell with the new larva was put into a test-tube, the glass side covering the hole.

The behaviour of the spinning larva inside the queen cell was observed through the glass; the larva could eat the royal jelly during the spinning process, as normally happens with the queen larva in the colony. Queen cells with normally developed larvae were placed in the suspended position, and queen cells with smaller larvae obliquely.

Female larvae fed with the mixed food of worker and drone larvae were placed either in queen cells or in a pupation dish similar to that described by Smith (1959). Filter paper was placed in the bottom of a Petri dish, which was then divided by waxed cardboard partitions into compartments, which were smaller than Smith's and similar to cells in a comb, or to queen cells. Some larvae were placed in muslin-lined Petri dishes. All the spinning larvae were kept in a desiccator chamber maintained at a relative humidity of 80%, which was then decreased.

The number of live larvae was recorded each day. Starting with series VI of queen 652, all larvae were weighed before they were transferred for pupation.

It was intended to determine the sex of surviving larvae as soon as possible; to get comparative material, larvae in worker and drone cells in normal hives were examined every day to find differences characteristic of the two sexes in live larvae.

Sex classification of the larvae reared was verified after partial or complete pupation.

RESULTS

Some points in the rearing technique were based on results of investigations concerning the behaviour of larvae in open and sealed cells, which will be dealt with elsewhere, together with a detailed description of some other results obtained during this work.

Survival rate of brood

Queen 266, and her daughters 580, 581 and 652, were chosen to produce brood which was reared and investigated for viability. Its survival rate is given in Table 1. About 50% of the brood of each queen survived. Previous histological investigations (Woyke, 1963*a*) showed that 50% females and 50% males hatched from eggs laid by these queens, and that in the hive the male larvae were eaten, mostly within 6–9 hours of hatching.

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

Queen No.	*No. eggs laid*	*No. larvae 5 days after egg laying*	*Percentage of larvae surviving*
266	280	136	48·6
580	not counted	scattered	about 50
581	213	112	52·3
652	196	108	55·1

Determination of the sex of live larvae

All the larvae (female and male) hatching from fertilized eggs laid by these queens were reared further. To establish the viability of the diploid drone larvae, it was necessary to determine by external signs the sex of each larva reared. It is easy to determine the sex of older larvae known to be of the same age, because drone larvae are smaller than female larvae up to the fifth day. This fact could not be used when the larvae were of different ages, or when they were in unusual conditions, as in the incubator, or while the rate of development of the diploid drones was still unknown. The sex could be determined only from the external rudiments of the genital organs and the sting, and published descriptions for fixed larvae (Michaelis, 1900; Zander, 1900; Zander, Löschel & Meier, 1916) could not be used, as characteristics look different in living and in fixed larvae. The outgrowths of the sting rudiments, and the lobes of the rudiments of external drone genital organs, are within the larval cuticle and not visible externally. Even the imaginal discs cannot be seen distinctly in the younger larvae. One white imaginal disc is visible on the ventral side of twelfth segment in a drone larva three days old, but the female larva also has such a disc on twelfth segment. The two pairs of imaginal discs on the ventral side of eleventh and tenth segments are only indistinctly visible. So we could not determine the sex of live larvae for certain until the third day. It was very easy to do so the next day, after the fourth moult, by a characteristic greenish-bluish fluorescence of all imaginal discs: of those on the ventral side of tenth, eleventh and twelfth segments of female larva, and of only one, on the ventral side of twelfth segment, of the male larva. The fluorescence of the imaginal discs decreased gradually in subsequent days.

Larvae reared in the incubator developed more slowly, and the fluorescence of the imaginal discs occurred on the fifth day of larval life. These sex characteristics were so unmistakable that verification of the sex in the pupal stage always confirmed the result obtained on the fifth day.

Viability of diploid drone larvae

All larvae hatched in the incubator from eggs laid by queens producing brood of low survival rate were reared until the fifth day without separating the sexes. Although histological investigations allowed us to suppose that 50% of all larvae reared were females and 50% males, the sex of each was not actually known until the fifth day.

At the beginning of the experiments, it was believed that larvae would show a difference in viability after 9 hours, because during that period most of the diploid drone larvae in the hive had been eaten. A lower viability of this 50% of larvae was expected, or even 50% mortality.

The number and percentage of larvae alive after 9 hours of rearing in the incubator are given in Table 2. An average of 99% were still alive, and in most of the series every larva was alive. There was no evidence of any lower viability of diploid drone larvae than of female larvae. Fifty-one of these larvae were fixed.

TABLE 2. Viability of brood of low survival rate after 9 hours of rearing in an incubator

Queen and series No.	*No. grafted larvae*	*Alive after 9 hr.*	
		No.	*%*
266 II	39	38	97
580	47	47	100
581 I	67	65	94
581 II	42	42	100
652 IV	47	47	100
652 V	54	54	100
Total	296	293	99·0

Most larvae were reared for 24 hours, and the number and percentage then still alive out of 656 hatching are given in Table 3. No clear decrease in the number of live larvae was found during this period. An average of 93·6% larvae were alive after 24 hours of rearing in an incubator. In many series (Table 4) *all* the larvae were still alive then. This was so especially in later series of experiments, when the technique of caring for the newly hatched larvae had been improved (Table 4).

Many of the larvae listed in Table 3 were fixed and investigated histologically; 50·7% out of 278 larvae were female and 49·3% males (Woyke, 1963*a*), and we may assume that the sex ratio among the remaining larvae (which were reared further) was similar. There was no evidence of lower viability of the diploid drone larvae within the first 24 hours of rearing in an incubator, yet during that time practically all the diploid drone larvae would have been eaten, had they been left in the hive. Results with 370 larvae left for further rearing are given in Table 4. Because larvae of the first series fixed for microscopical investigation are excluded here, a survival rate of 95·9% was obtained for brood reared for 24 hours in the incubator; this higher percentage was due to the improved treatment of the newly hatched larvae in the later series of experiments.

TABLE 3. Viability of brood of low survival rate after 24 hours of rearing in an incubator

Queen and series No.	*No. grafted larvae*	*Alive after 24 hr.*	
		No.	*%*
266 I	91	84	93
266 II	39	37	95
580	12	9	75
581 I	51	47	91
581 II	42	41	98
581 III	32	31	97
652 II	82	68	83
652 III	23	22	96
652 IV	47	47	100
652 V	54	54	100
652 VI-XVI	183	174	94
Total	656	614	93·6

TABLE 4. Viability of brood of low survival rate on subsequent days of rearing in an incubator

Queen and series No.	*No. grafted larvae*	*Percentage of live larvae on successive days*				
		1	2	3	4	5
266 I	27	93	85	70	48	37
266 II	39	95	90	87	80	72
581 I	13	92	92	92	92	92
581 II	42	98	93	83	76	67
652 IV	36	100	100	100	97	86
V	30	100	90	77	70	50
VI	9	100	100	100	56	44
VII	12	100	100	100	100	83
VIII	23	100	65	61	61	52
IX	29	100	100	97	86	86
X	34	88	79	59	53	50
XI	15	87	87	73	67	67
XII	7	100	71	57	57	57
XIII	6	100	83	67	67	67
XIV	12	92	92	92	83	67
XV	32	94	88	88	84	78
XVI	4	100	100	75	75	75
Total No.	370	355	330	291	276	246
% still alive	100·0	95·9	89·2	78·6	74·6	66·5
% daily mortality	0·0	4·1	6·7	10·6	4·0	8·1

Some dead larvae were found on subsequent days, amounting to 4·0–10·6%. The average was 6·6% per day, with no striking increase in mortality on any one day (Table 4).

The sex of larvae reared was not known until the fifth day, but since 74·6% still survived on the fourth day, it was known that some must be male. Even on the fifth day, more than 50% were still alive in 13 out of the 17 series.

On the fifth day sex determination was easy and certain. Results are given in Table 5. Drone larvae were found in all series except one small one, where 4 out of 7 larvae survived. Of the total number of larvae alive on the fifth day of rearing, 49·2% were female and 47·6% were male, 3·2% not being distinguished. Roughly equal numbers of females and males must have died. It can be seen from Table 5 that more males than females survived in many series in which the loss of larvae was especially high, suggesting that in unfavourable conditions diploid drone larvae are more viable than female larvae.

TABLE 5. Sex of brood of low survival rate after five days of rearing in an incubator

Queen and series No.	*No. grafted larvae*	*Larvae alive after 5 days*		*% of each sex*		
		%	*No.*	*female*	*male*	*unknown*
266 I	27	37	10	40	50	10
266 II	39	72	28	43	57	
581 I	13	92	12	42	58	
581 II	42	68	28	46	50	4
652 IV	36	86	31	68	32	
V	30	50	15	27	47	26
VI	9	44	4	50	25	25
VII	12	83	10	50	50	
VIII	23	52	12	42	58	
IX	29	86	25	60	40	
X	34	50	17	35	59	6
XI	15	67	10	30	70	
XII	7	57	4	100	0	
XIII	6	67	4	50	50	
XIV	12	67	8	50	50	
XV	32	78	25	60	40	
XVI	4	75	3	33	67	
Total	370	66·5	246	49·2	47·6	3·2

All the results showed a viability of diploid drone larvae no lower than that of female larvae produced by the same queen. In the hive the diploid drone larvae thus have the same *viability* as the diploid worker larvae which the bees rear to maturity.

The female larvae were transferred the next day (6th–7th) to sealed queen cells or to a pupation dish. The drone larvae were put on to new food daily, and until the 9th–10th day, when they were transferred to a pupation dish.

Influence of rearing conditions on larval development

It is believed that the greatest difficulty in rearing larvae in the laboratory lies in maintaining the right humidity and in supplying the right food to the larvae.

Larvae kept all the time at 100% relative humidity did not develop well, and lost their white appearance. Much better results were obtained at 96%, but this was too dry for the bee milk of larvae less than one day old, which dried at that humidity, both in queen cells and in worker cells. The best results were obtained when newly hatched larvae transferred to the bee milk of the youngest larvae (in worker or queen cells) were kept at 100% relative humidity for the first day, the humidity being decreased to 96% next day, and to 80% after the larvae were transferred to sealed queen cells or pupation dishes. After the pupation was completed, the relative humidity was decreased to 60%.

In the hive, the bee milk supplied to the larva just hatching is transparent and water-white, thus differing from the white bee milk supplied later; this water-like milk seems necessary for the hatching larvae.

The larvae reared developed much better when the newly hatched larvae were transferred first on to royal jelly or younger worker food. Both foods were taken from cells containing larvae not more than 24 hours old. Larvae fed all the time with royal jelly put by bees into queen cells during only one day did not develop well, even if larvae two days old were grafted into the cups in the hive on the previous day. Larvae in the incubator supplied with this food grew well, but they lost their white appearance and became brownish. None of these larvae pupated successfully. The best results with royal jelly were obtained after very young larvae were grafted into the cups in the hive, to provide royal jelly corresponding to larvae of the same age as the larvae reared in the incubator. Transferring larvae to mixed worker food after the fourth day gave very good results. The development of larvae reared in the incubator was one, sometimes two, days behind that of larvae raised in the hive by the bees.

The influence of different foods on larval development is shown by the weight reached by larvae being transferred to pupation; this was determined for the last ten series. The 33 female larvae fed only with royal jelly reached an average weight of 173·6 mg., compared with 261·5 mg. for 15 larvae fed in the later days with mixed food of worker larvae; 15 drone larvae fed only with royal jelly reached an average weight of 199·1 mg. compared with 406·1 mg. for 13 larvae fed in the later days with mixed food of worker larvae. Probably royal jelly contains a higher concentration of growth inhibitor or other substance necessary to stimulate pupation. It was very easy to rear gigantic individuals on mixed food of older worker larvae, for instance female larvae of 385 mg. and drone larvae of 500 mg. (62·5 and 115 mg. respectively higher than the maximum weight reached by larvae fed normally in the hive). Larvae in the hive fed with mixed food do not grow further, probably because they do not get the requisite *amount* of food.

Many drone larvae fed only with royal jelly developed badly, and only those reaching a weight of more than 310 mg. pupated successfully. Nevertheless some adult drones of normal size were reared on royal jelly alone.

The weights quoted above are not the *maximum* larval weight, which is known to be higher than the adult weight. In the circumstances described, however, larvae reaching a weight of say 126 and 135 mg. at the time of transference to sealed queen cells developed into adults weighing 154 and 162 mg. respectively.

The weight of female larvae 6–7 days old and of drone larvae 9–10 days old cannot be compared with weights at the same age in the hive, since their development lagged one or two days behind that of bees raised in the hive.

Rearing male and female diploid adults

All the brood was reared further than so far described. But it became difficult to compare the viability of diploid male and female larvae on successive days. The females were transferred earlier to pupation dishes, and they finished their development earlier. Individuals in the sealed queen cells mostly did not need any help, but some of the smaller larvae in queen cells, and some of the larvae in the larger compartments of pupation dishes, needed some help. If the tip of the abdomen was not fastened to the wall of the cell or compartment with the faeces, the larval cuticle was not cast completely at pupation, but remained (folded) attached to the tip of pupal abdomen. A cuticular layer also remained in the proctodeum. In such cases it was necessary to remove the larval cuticle from the abdomen and proctodeum of the pupa. Queens emerged easily from the sealed queen cells, but intermediates had difficulty in opening the cell capping.

Adults of both sexes were reared. It is interesting that most female larvae fed all the time with royal jelly gave only queens, with some intermediates; contrary to the literature, no workers were obtained. Our results are probably due to the use of the right food for each stage of larval development. Workers developed from larvae fed in the later days with mixed food of older worker larvae.

The most interesting result is that some adult drones were reared from fertilized eggs. A description of these drones will be given in another paper, together with cytological and genetical evidence of their diploidy. Larvae, pupae and adult drones were all reared in incubators from brood of 'low survival rate', which would have been eaten by the bees if left in the hive.

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