AM. ZOOLOGIST, 4:111-123 (1964).

BEHAVIOR GENETICS OF NEST CLEANING IN HONEY BEES. IV. RESPONSES OF F_1 AND BACKCROSS GENERATIONS TO DISEASE-KILLED BROOD

WALTER C. ROTHENBUHLER

Department of Zoology and Entomology, The Ohio State University

INTRODUCTION

The honey bee is in one striking way different from most animals. Much of the animal kingdom lives a more or less solitary existence (except for a minimal amount of social life involved in mating activity and reproduction), whereas the honey bee lives in colonies of up to 60,000 individuals. Honey-bee colonies are much more than aggregations, flocks, or herds, for the individuals within one are so dependent upon one another that no individual, nor even a group of a few hundred individuals, can survive and perpetuate the species in nature.

Some further facts of bee biology are basic to this paper. The nest is the center of colony life, and is composed of a number of wax combs arranged vertically in a suitable cavity such as a hollow tree. In modern apiculture, hives, instead of hollow trees, provide the cavities, and combs are contained inside wood frames which permits their removal from the nest, their examination, and rearrangement. Brood is reared and food is stored in these combs which are used over and over again, year after year.

The drone and the queen (male and female) are the reproductively important members of the colony. Drones develop from unfertilized eggs, and are consequently haploid in origin and in transmission of genetic factors. Queens arise from fertilized eggs, which makes them diploid in origin and similar to any diploid animal in transmission of genetic factors. The third kind of bee in the colony, the worker bee, is a reproductively undeveloped female which arises from a fertilized egg, the same as the queen.

Colony behavior is almost exclusively the behavior of worker bees. It is the worker bees that secrete wax and build comb, nurse the brood, keep the colony warm or cool as the need may be, defend the colony, keep the combs and hive interior clean, and go to the field to gather nectar, pollen, water, and propolis. Such colony labor is further divided among worker bees on the basis of their age. Normally the younger workers engage in duties inside the hive whereas older workers carry out the field activities. By this time, it is perhaps apparent that a social insect such as the honey bee displays a far greater variety of behavior than could be expected of insects living a solitary life. More information on life history, behavior, and social organization of honey bees may be found in Butler, 1954; Von Frisch, 1950; Grout, 1963; Lindauer, 1961; Michener and Michener, 1951; Ribbands, 1953; Root, 1962; as well as in a host of other sources.

The stock-in-trade of the behavior geneticist, however, is variation in behavior, and the honey bee has it. Different races and strains of bees vary, for instance, in the number of times their colonies swarm, in the number of times they sting, and in the amount of pollen stored. They even engage in different dances in reporting the same food source to the colony (Boch,

The data contained in this paper were obtained while the author was a staff member of the Department of Zoology and Entomology, Iowa State University, Ames, Iowa. In addition to support from the Iowa Agricultural Experiment Station, this investigation was supported in part by Research Grant E599(C7) from the National Institute of Allergy and Infectious Diseases, Public Health Service, made to Iowa State University, and is presently supported by Research Grant HD00368-01 from The National Institute of Child Health and Human Development, Public Health Service, made to The Ohio State University.

The author's deep appreciation is expressed to Mrs. Christina Barthel (nee Garwood), technician, and Mr. Victor C. Thompson, research associate, for their competent assistance with these experiments.

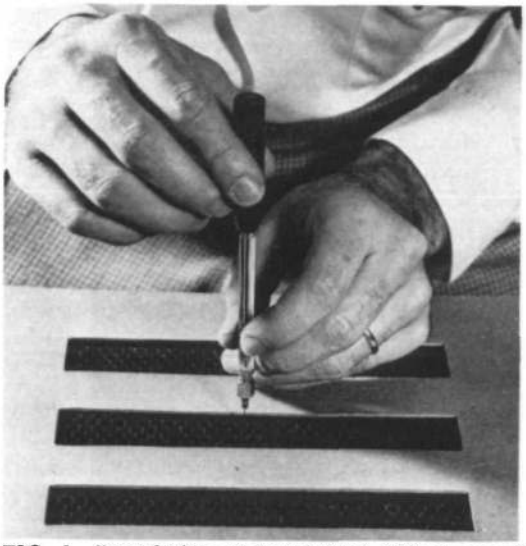

FIG. 1. Inoculation of larval food with spores of *Bacillus larvae* by use of microsyringe.

1957; and others). There is a great deal of behavioral variation in bees but a great lack of careful, quantitative investigation of this variation. More study in this field would be rewarding.

Our studies have been concerned primarily with two differences in behavior. The first one is response to immature individuals which have died, in the cells of the brood comb, of the disease American foulbrood; and the second one, studied to a lesser extent, is response to the beekeeper —more specifically, stinging behavior.

NEST-CLEANING BEHAVIOR

Description and nature of investigation

The adults of certain colonies of bees remove foulbrood-killed larvae and pupae from the combs very quickly, whereas the adults of other colonies allow these dead individuals to remain in the combs for days, or weeks, or indefinitely. Such larvae and pupae, dead of American foulbrood, allowed to remain in the brood nest are a source of the pathogen, *Bacillus larvae*, and lead to new infections. This hygienic behavior, by which dead larvae and pupae are quickly removed, is one mechanism of resistance to the disease (Rothenbuhler and Thompson, 1955).

Differences in response to American foulbrood-killed brood were discovered more than 20 years ago by O. W. Park (1936) at Iowa State College, and by Alan Woodrow and E. C. Holst (1942) of the United States Department of Agriculture at Laramie, Wyoming, as they were investigating the mechanisms of resistance to disease. The research reported here was undertaken as part of our investigation of resistance to American foulbrood, but it has grown into a new effort on behavior genetics of bees (Rothenbuhler, 1958, 1960, 1964; Jones and Rothenbuhler, 1964; Thompson, 1964).

Hygienic behavior may be studied by techniques indicated in Figures 1, 2, and 3. A sample of eggs from a suitable mated queen is obtained in a comb, and is placed for hatching and rearing in the colony whose behavior is to be studied. At the appropriate larval age, the larval food sur-

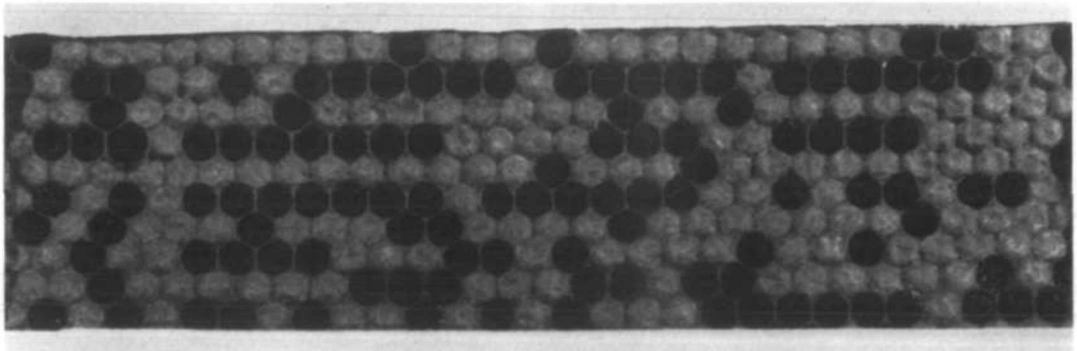

FIG. 2. Comb of brood, reared in a Brown, hygienic colony, about two days before brood emergence, showing that many individuals are missing from the spore-inoculated rows. All brood remaining in this comb was found to be alive.

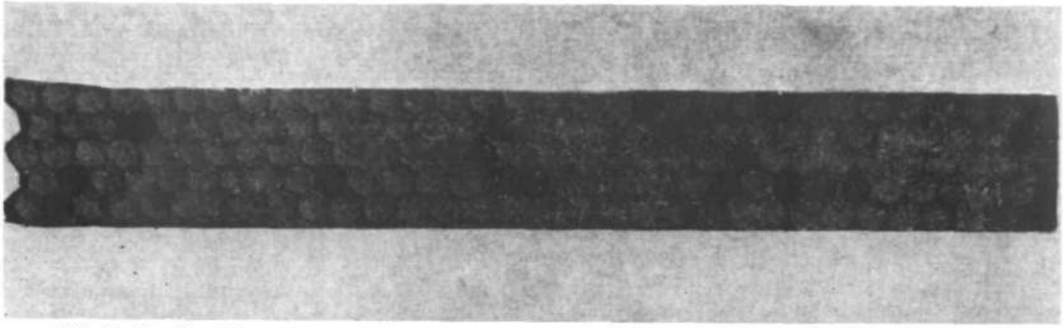

FIG. 3. Comb of brood, reared in a Van Scoy, non-hygienic colony, showing that most of the brood seems to be present. When uncapped in the laboratory, many individuals in the spore-inoculated rows were dead of American foulbrood.

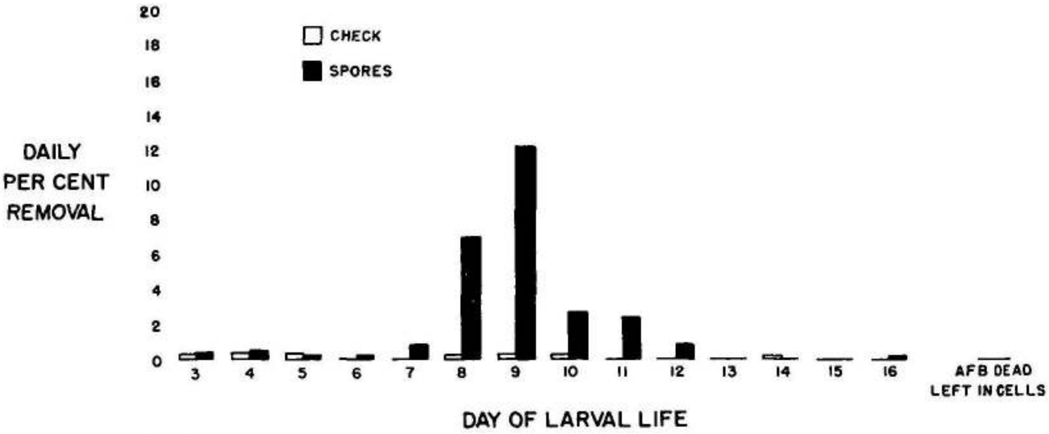

FIG. 4. Behavior of three Brown colonies resulted in the removal of all American foulbrood-killed (AFB) individuals before the end of the experiment.

rounding each larva is inoculated, by means of a microsyringe, with spores of the pathogen dispersed in water. Control (or check) larvae are inoculated with water only, or are uninoculated, as the situation requires.

Figure 2 is a picture of a comb photographed at the end of an experiment. It shows spore-inoculated rows of brood from which many individuals have been removed as contrasted with the check rows of brood. This brood was reared in a colony showing hygienic behavior. Figure 3 is a picture of brood reared in a colony which did not show hygienic behavior. Nearly all individuals seem to be present, but when these cells were manually uncapped, large numbers of larvae were found to be dead of American foulbrood in the spore-inoculated rows.

Experimental results

Figures 4, 5, and 6 show graphs of the data from an experiment involving colonies from our Brown resistant line, our Van Scoy susceptible line, and the F_1 generation resulting from a cross of the two. All tests were made concurrently.

Figure 4 shows the pooled daily percentage removals of check and spore-treated larvae from three Brown-line colonies. On the first day of larval life, not shown in the graph, the larvae were inoculated. On the second day of larval life the larvae were counted and this was the base count for the subsequent calculations. On the horizontal axis it can be seen that, by the third

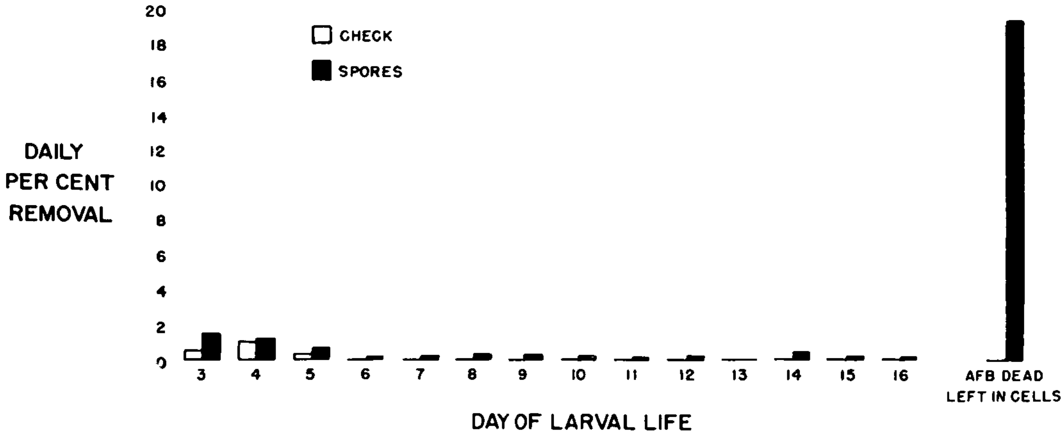

FIG. 5. Behavior of four Van Scoy colonies left most foulbrood-killed individuals in the comb until the end of the experiment on the 16th day.

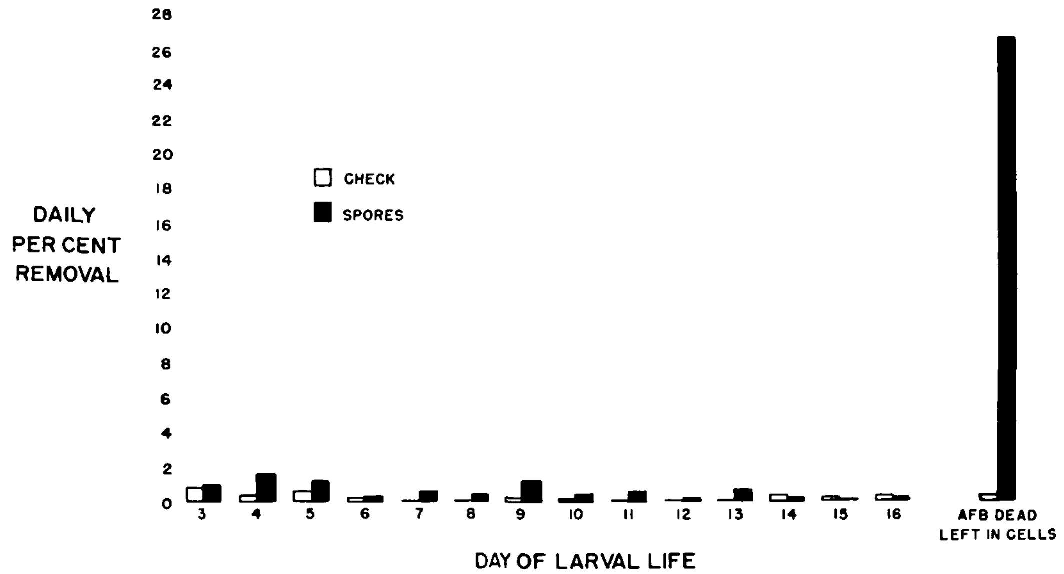

FIG. 6. Behavior of five F_1 colonies resembled the behavior of the Van Scoys.

day, a small percentage of the base count of checks was removed, and also a small percentage of the spore-inoculated base count. It may be pointed out here that capping of the cells occurs between the 4th and 6th days, and the larval stage ends by about the 8th day. There was a substantial amount of removal of spore-inoculated larvae on the 8th, 9th, 10th, and 11th days, and no substantial removal of checks. Very little removal occurred on the last days of the experiment. On the 16th day, at the end of the experiment, all cells were uncapped manually and no individuals dead of American foulbrood were found remaining in the combs. This graph is based upon 622 spore-fed larvae and 322 water-fed larvae.

Figure 5, presenting the pooled performance of four Van Scoy susceptible-line colonies, shows a contrast. Practically no removal of brood occurred throughout the experiment, and at the end, when the cells were uncapped, many of the spore-inoculated larvae were found dead of American foulbrood and remaining in the comb.

This graph is based upon 753 spore-fed larvae and 383 water-fed checks.

The pooled performance of five F_1 colonies, presented in Figure 6, was very close to the Van Scoy susceptible-line performance. Here again a large number of larvae were involved—837 spore-fed, and 459 water-fed. These results indicate that the gene or genes for hygienic behavior were recessive. How many loci are involved is now a prominent question.

To get an answer, 29 backcrosses of the F_1 generation were made. For the preceding test, F_1 workers had been reared, but they do not normally reproduce. F_1 queens were necessary for reproduction. From such F_1 queens, drones were obtained. These drones, having developed from unfertilized eggs were gametes—gametes with wings, in fact, and the capability of producing 6 to 10 million more genetically identical gametes in the form of sperms. Such a gamete, from an F_1 individual, when mated by artificial insemination to an inbred queen of the parental line, will produce a colony of bees of similar genotype. Twenty-nine such drones or gametes from two queens of the F_1 generation were mated to 29 queens of the Brown line which were expected to be homozygous for the recessive genes for hygienic behavior. If $1/2$ of the 29 colonies developed from the backcrosses were hygienic, one would conclude that one locus only was involved in the difference in hygienic behavior between the two lines. If only $1/4$ were hygienic, 2 loci would be involved; if $1/8$, three loci. It was necessary, of course, to run controls along with the backcrosses, so 7 colonies from the hygienic Brown line were tested, along with 7 colonies of the non-hygienic Van Scoy parental line. Also 8 backcrosses were made to the Van Scoy line.

Figure 7 presents graphs of the hygienic behavior of the seven hygienic-line control colonies and a graph of the pooled results. The pooled data in the upper left corner involve a total of 1383 spore-fed larvae and 698 water-fed checks. The same pattern seen previously in the Brown line is obvious. Not a single dead individual remained beyond the 12th day. An unexpectedly high number of check larvae were removed. There is no reason to think that these were dead; in fact it is likely that they were living larvae that for some reason were removed, and consequently they present a problem for the future. The individual responses of the seven colonies are plotted in the seven other graphs which show great uniformity with respect to the main point—that Browns remove foulbrood quickly.

Figure 8 shows seven cases of Van Scoy non-hygienic behavior, with the pooled results again, in the upper left. This too resembles the non-hygienic behavior already seen. Here it is seen that there was only a slight amount of removal of either spore or check larvae. In the end, about 14% of spore-fed larvae were left dead in the cells of the comb. Less than .5% of the check larvae were dead and left. A glance at the seven individual-colony results shows that all colonies left foulbrood-killed brood in the comb. Some colonies removed more spore-fed larvae than others, but this removal was erratic as to when it occurred.

The 29 backcrosses to the Brown line broke up into 4 groups, each of which is presented in a separate figure. Figure 9 presents a group of 6 colonies that showed complete hygienic behavior. Five of the 6 showed behavior nearly identical to the Brown line. M 1423 was about 2 days later in removal than expected, but no dead larvae remained. Six colonies constitute about $1/4$ of 29, which suggests that 2 loci are involved.

None of the other 23 colonies showed hygienic behavior, but Figure 10 presents some results that were unexpected. Nine colonies left uncapped dead larvae in the combs. In fact the caps on cells containing dead brood had been removed in every case except for 1 cell in the M 1428 colony and about half such cells in the M 1444 colony. This result was in contrast to the remaining 14 colonies, all of which retained dead brood in capped cells.

There is some variation in the colonies of Figure 10. M 1416, in the upper left, removed to a considerable extent. Perhaps it belongs in the hygienic group, but it did not show complete hygienic behavior. The next seven cases seem to be more surely uncappers and non-removers. M 1444, in the lower right, was not completely uncapped at the end of the experiment but seems to belong in this group, nevertheless.

In addition to the indication of two loci from the proportion of hygienic colonies, the 1/4 that uncapped suggests that one locus pertains to uncapping. If there is one locus for uncapping in a two-locus situation, the other one should be a locus for removing. This idea can be tested by examining the 14 remaining colonies to see whether or not they will remove dead larvae following human uncapping.

Figure 11 shows a group of 6 colonies that showed very little hygienic behavior during the course of the experiment and

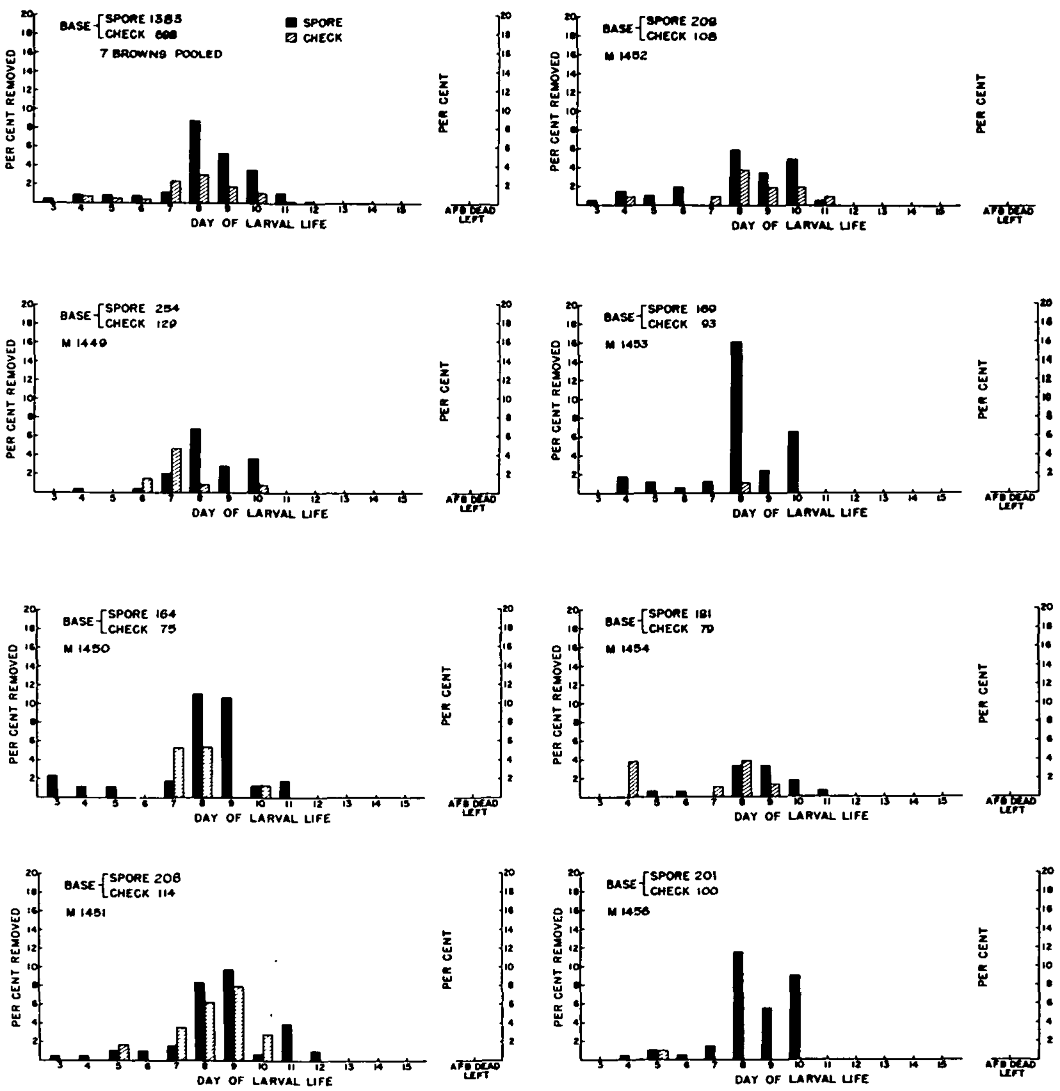

FIG. 7. Responses of seven, Brown-line, control colonies to the indicated numbers of spore-inoculated and check-treated larvae. Data from the seven different matings have been pooled in the upper left graph.

retained a lot of dead brood. When the combs containing foulbrood-killed individuals were uncapped by us, reassigned to different colonies, and placed in them for testing, the colonies removed a large percentage of the dead brood in 2 days. As can be seen in Table 1, M 1412, M 1415, and M 1431 removed more than 90% in two days. The other three ranged from 53 to 75%. All of these colonies removed more on the second day of the removal test than on the first, indicating perhaps, that worker bees were being increasingly attracted to the job.

The last group of 8 colonies (Fig. 12) showed very little hygienic behavior during the course of the experiment, and removed fewer uncapped individuals than the preceding group during the second test (Table 2). Matings 1429, 1432, and

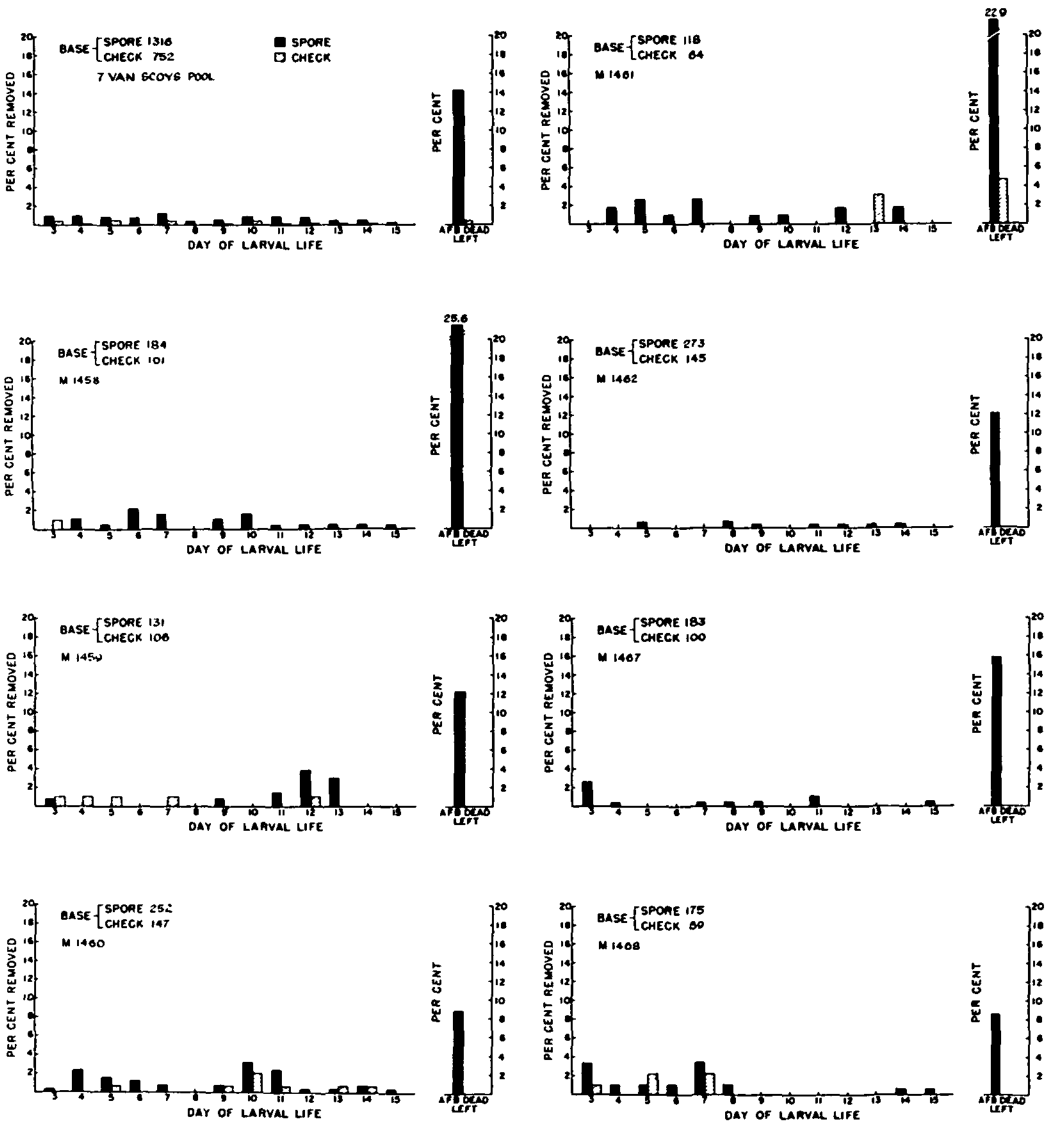

FIG. 8. Responses of seven, Van Scoy-line, control colonies to the indicated numbers of spore-inoculated and check-treated larvae. Data from the seven matings have been pooled in the upper left graph.

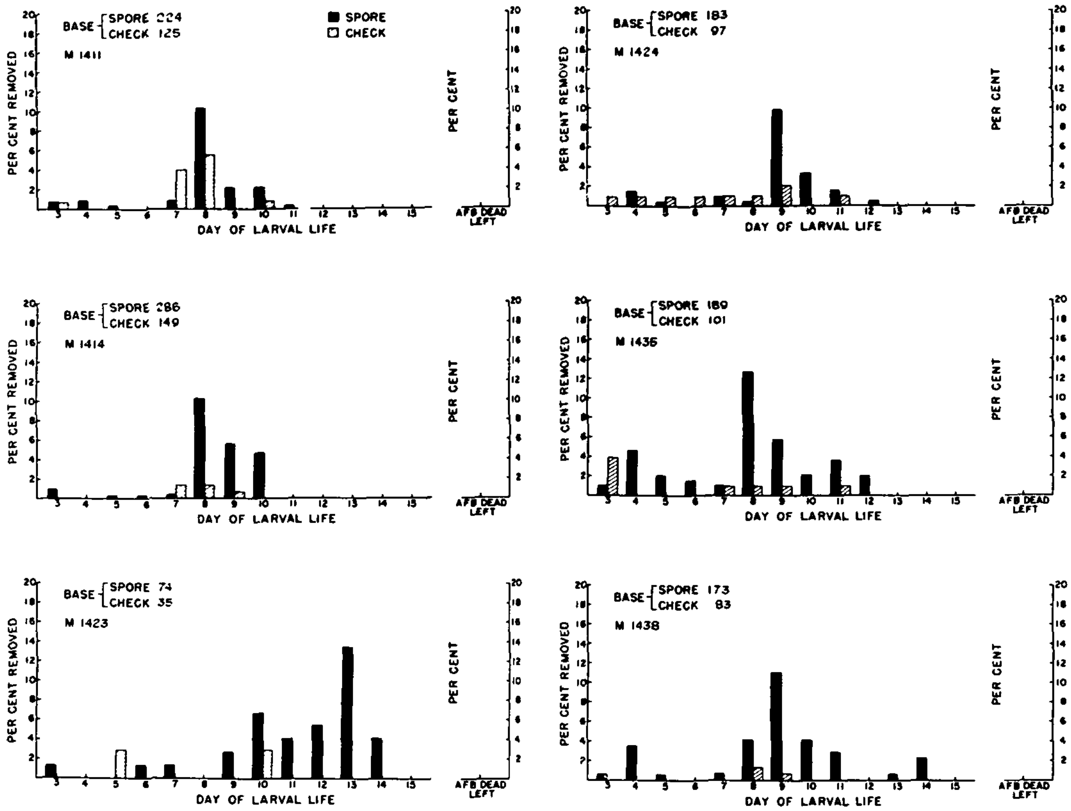

FIG. 9. Hygienic responses of 6 of 29 colonies developed in each case from a backcross of one drone from an F_1 queen to a Brown-line queen.

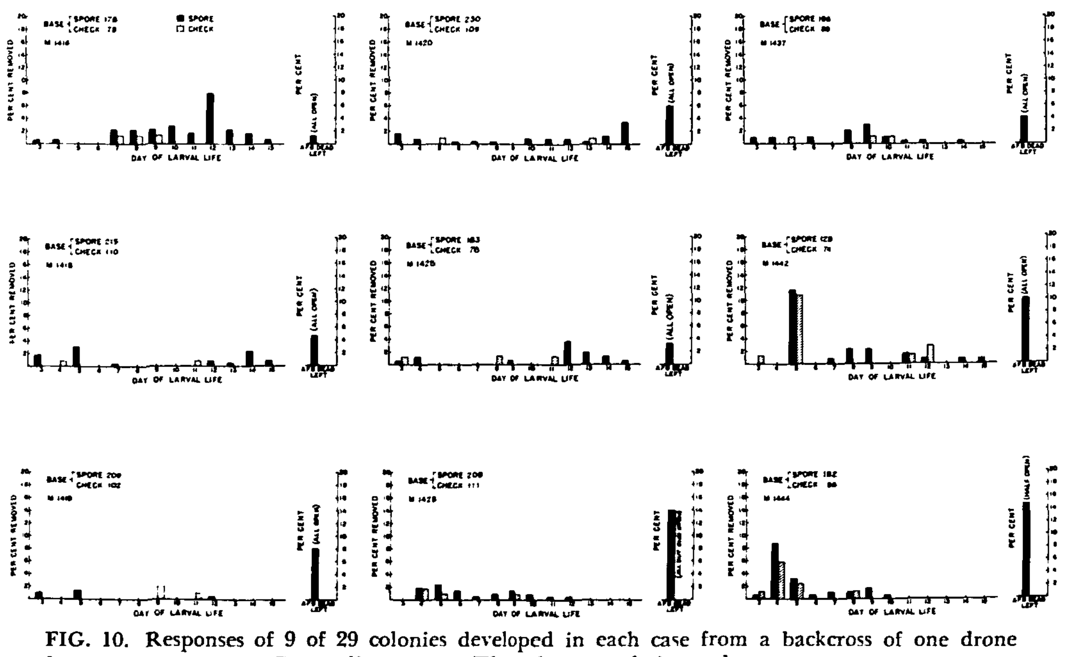

FIG. 10. Responses of 9 of 29 colonies developed in each case from a backcross of one drone from an F_1 queen to a Brown-line queen. These bees are designated as uncappers.

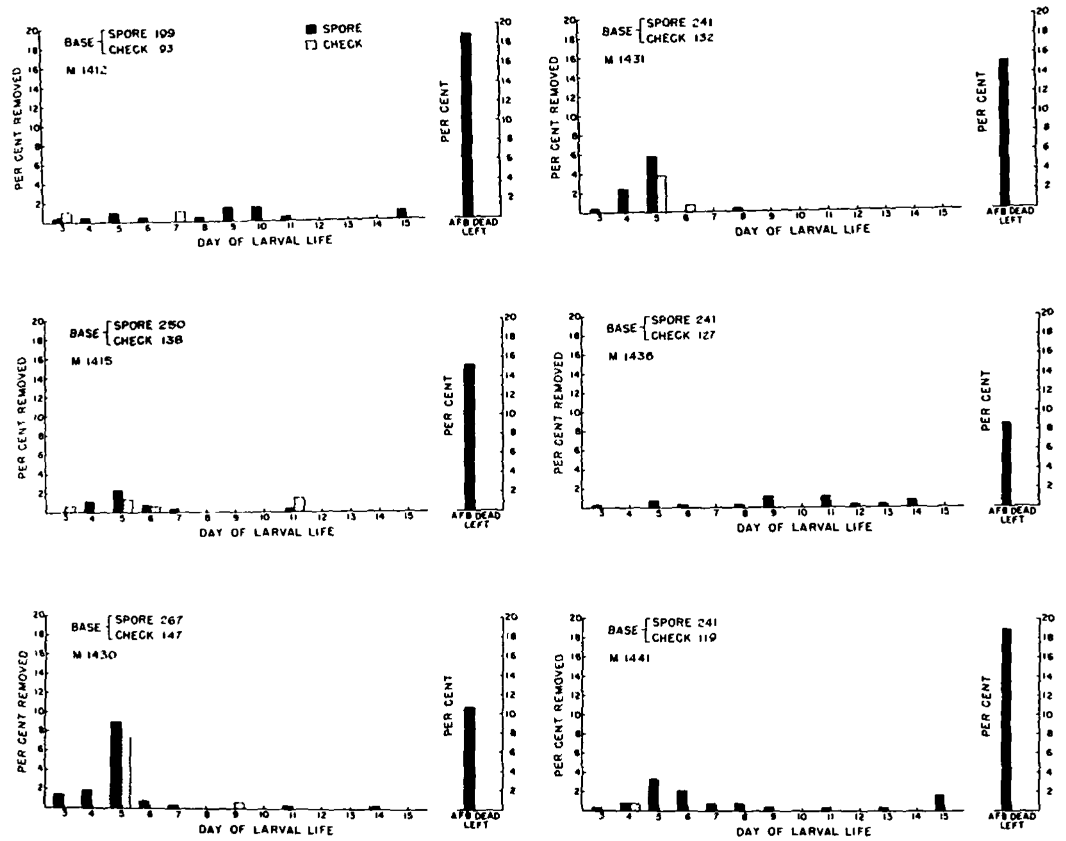

FIG. 11. Responses of 6 of the 29 backcross colonies. This group did not uncap, but did remove following human uncapping.

1434 did not remove a single dead individual, and M 1443 removed 3% in 2 days time. The other colonies ranged from 15 to 47% total removal.

Although this group of 14 colonies is not as discretely separated into removers and non-removers as the whole group of 29 was separated into uncappers and non-uncappers, for the time being the 14 are interpreted as falling into two groups. This seems to be the simplest possible explanation and justifiable in the light of inadequate knowledge about the environmental factors that affect removal rate.

The backcrosses of sons of F_1 queens to the Van Scoy-line queens present something of a problem. None of these colonies

TABLE 1. *Percentage of dead individuals removed from uncapped comb on each day based on number present at beginning of day, and percentage of total sample removed.*

M. No.	Dead individuals	Per cent removed: 1st day	2nd day	Total
1412	47	70	71	91
1415	38	63	79	92
1430	30	13	46	53
1431	38	50	89	95
1436	16	25	67	75
1441	29	24	55	66

TABLE 2. *Percentage of dead individuals removed from uncapped comb on each day based on number present at beginning of day, and percentage of total sample removed.*

M. No.	Dead individuals	Per cent removed: 1st day	2nd day	Total
1413	33	9	33	39
1417	38	29	26	47
1429	18	0	0	0
1432	21	0	0	0
1433	22	0	23	23
1434	41	0	0	0
1440	32	6	10	16
1443	39	3	0	3

was expected to show hygienic behavior because Van Scoy queens were expected to be homozygous for dominant genes for non-hygienic behavior. As Figure 13 shows, M 1409 showed a typical pattern of hygienic behavior. This result is beyond explanation at present. No F_1 colony has ever displayed complete hygienic behavior and in this case the backcross of an F_1 to the Van Scoy line does so. We cannot disregard this result, regardless of how much we would like to, but we are basing the genetic hypothesis on the other data.

Hypothesis

From the foregoing, the hypothesis presented in Figure 14 has been developed. Hygienic behavior (in the upper left corner) depends upon homozygosity for two recessive genes—one a gene (designated u) for uncapping of cells containing dead larvae, the other a gene (designated r) for removal of dead larvae. A cross between

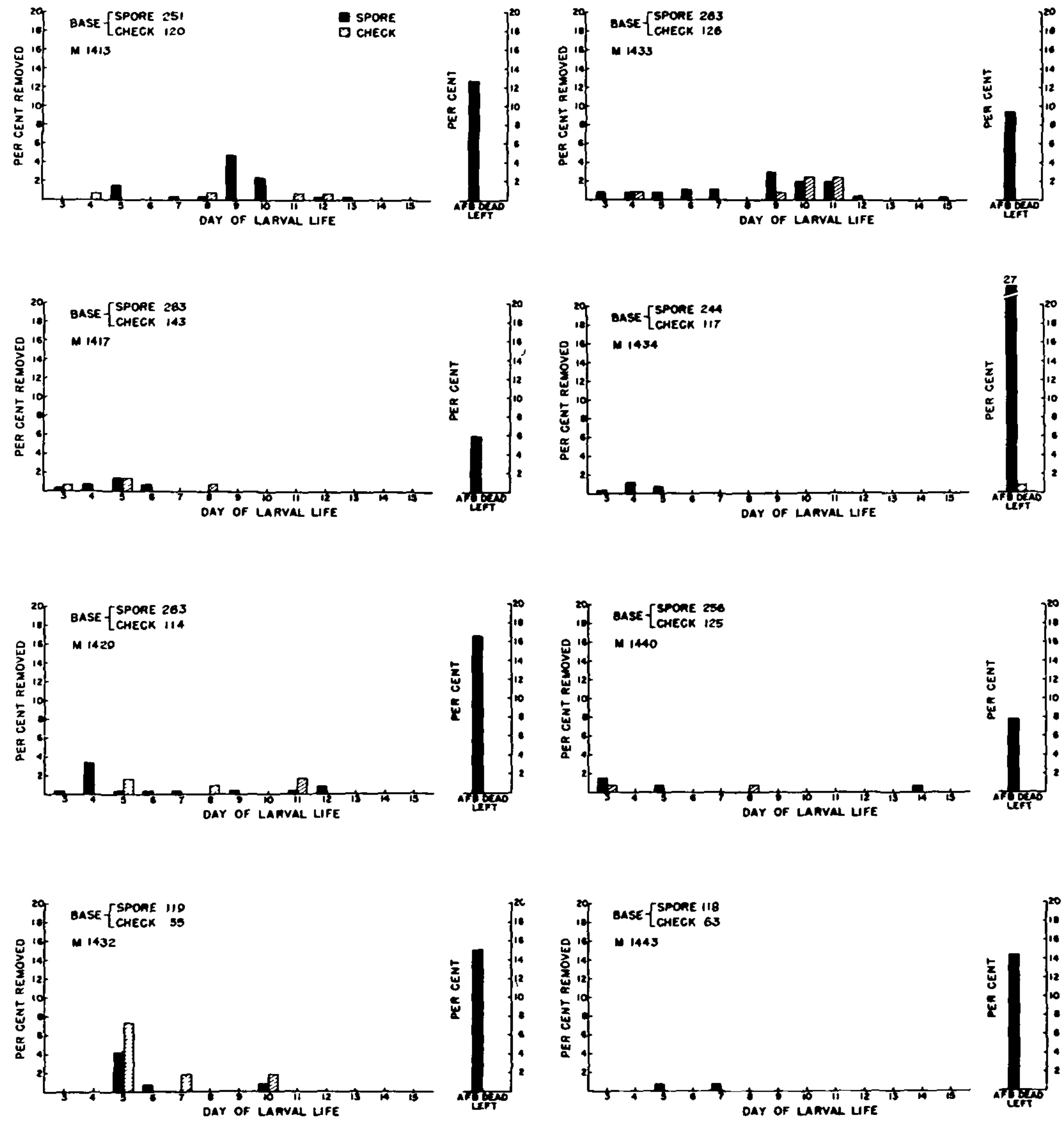

FIG. 12. Responses of the 8 backcross colonies which neither uncapped nor removed.

the two opposite homozygous types would result in an F_1 which is non-hygienic. An F_1 queen would produce four kinds of drones or gametes which, backcrossed to the parental type showing hygienic behavior, would result in four kinds of backcross colonies in a 1:1:1:1 ratio. In this case a 6:9:6:8 ratio was obtained.

One of the most attractive features of this hypothesis is the ease with which it can be tested. One can predict how each of these classes should behave in further breeding. Queens and drones from the hygienic group should breed true for hygienic behavior. The uncapping group should breed true for uncapping, and the removing group for removing. For the time being, however, most effort is going into studies of the environmental factors that may conceivably affect the colony's response to dead brood. For these investigations, the parental lines have been and are being used. It is equally important to test the F_1 generation for its stability and variability under various environmental conditions.

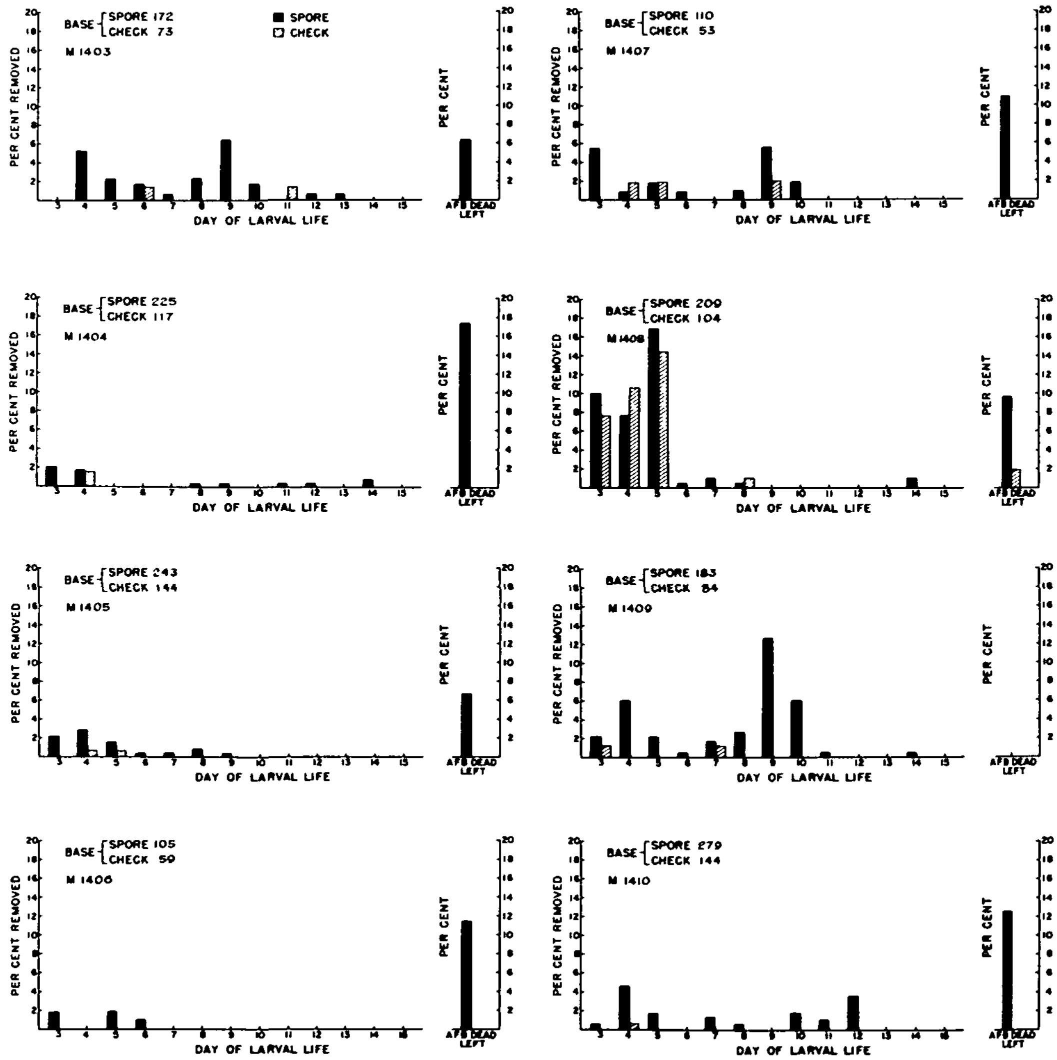

FIG. 13. Responses of 8 colonies developed in each case by backcrossing one drone from an F_1 queen to a queen from the Van Scoy line.

TABLE 3. *Frequency distribution of four groups of colonies according to the total number of times the beekeeper was stung while engaged in the same operations with each under similar conditions. Fourteen visits were made to each colony.*

	Total number of stings																
Type of colony	0	1	2	3	4	5	6	7	8	11	15	19	20	21	23	26	31
Seven Van Scoy colonies—None hygienic	6	1															
Seven Brown colonies—All hygienic										1	1	1	1	1		1	1
Twenty-nine colonies from backcrosses of F_1 to Brown line—Six hygienic	9	9	2	3	1	2		1	1						1		
The six hygienic colonies from above backcrosses	2	3						1									

STINGING BEHAVIOR

In the hygienic behavior experiment, observations on several other characteristics were made. One of the most obvious was *stinging* behavior. Table 3 presents the data. In the course of 98 visits to the 7 Van Scoy colonies, we were stung only once. In the course of 98 similar visits on the same days to the 7 Brown colonies, we were stung 143 times. The fact that this was an obvious characteristic needs no emphasis, and it may be said that sophisticated quantitative measurements were not really necessary to alert us that we had a real, line difference.

It has been thought by most people that disease-resistant bees are always cross, and logically explained further that keeping the brood nest free of dead larvae and defending the colony against the beekeeper is a manifestation of the same general characteristic—a high level of vigor in the bees.

If hygienic behavior and stinging behavior are due to the same underlying characteristic, namely vigor, the hygienic colonies among the backcrosses ought to be stingers. This was not so. There were 2 hygienic colonies that never stung, 3 stung once each, and one stung 7 times. The colony that stung equally as much as the parental, hygienic line was non-hygienic. The stinging behavior of all 29 of the backcross colonies indicates that more than one or two loci are involved in this behavior difference.

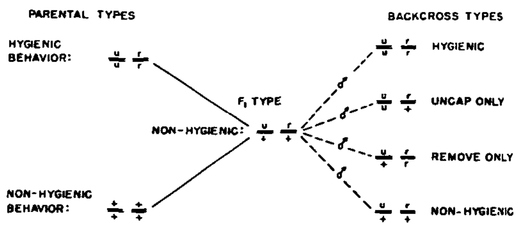

FIG. 14. Genetic hypothesis offered in explanation of different responses to AFB-killed brood observed in 63 colonies of bees.

In these experiments, colony behavior characteristics which appeared together in the parental lines have separated in the backcross colonies. We are accustomed to this result among individuals, but here it is happening among colonies made up of many individuals. This can occur only when the individuals making up the colony are genetically similar, and they can be genetically similar only when the mother of the colony is highly inbred and has been mated to a single haploid drone. With the exception of the sex locus, the mother makes identical (or nearly identical) genetic contributions to every worker bee, and the father's contribution is expected to be genetically identical in every case. This *inbred queen - single drone* technique promises to be useful in the analysis of colony behavior characteristics (Rothenbuhler, 1960).

SUMMARY

Two lines of honey bees differ greatly in the response of their colonies to brood killed by the disease American foulbrood and in the number of times they sting the

beekeeper during a colony inspection. The Brown line removed dead larvae quickly (hygienic behavior), and in one experiment in which counts were made, stung the beekeeper about 1.5 times per colony visit. The Van Scoy line left dead larvae in the brood nest throughout the course of the experiment and stung the beekeeper about 0.01 times per colony visit.

The F_1 generation allowed dead larvae to remain in the brood nest as did the Van Scoy line. No observations were recorded on the stinging behavior of the F_1.

A technique of mating single drones from F_1 queens to inbred queens of the original lines was employed to secure colonies with genetically similar worker bees in each for colony tests. Colonies resulting from such backcrosses to the Brown line were of four types: (1) uncappers of cells and removers of dead brood contained therein, (2) uncappers only, (3) removers only after human uncapping, (4) neither uncappers nor removers.

From these results a two-locus hypothesis has been developed. It states that uncapping of a cell containing dead brood is dependent upon homozygosity for a single recessive gene (designated u), and removing is dependent upon homozygosity for a single recessive gene (designated r). The hypothesis can be tested by breeding and investigating further generations within each phenotypic class, but before this is done, information is needed on the effect of certain environmental factors. Such information is being collected presently.

The same backcrosses that were tested for hygienic behavior indicated that stinging behavior is dependent upon more than one or two loci. Stinging behavior and hygienic behavior assorted independently in the backcross colonies. This result shows that the two behaviors depend upon different genetic bases.

REFERENCES

Boch, R. 1957. Rassenmassige Unterschiede in den Tänzen der Honigbiene (*Apis mellifica* L.). Z. vergleich. Physiol. 39:289-320.

Butler, Colin G. 1954. The world of the honeybee. Collins, St. James Place, London.

Frisch, K. von. 1950. Bees, their vision, chemical senses, and language. Cornell University Press, Ithaca, New York.

Grout, Roy A. (ed.). 1963. The hive and the honey bee. Dadant & Sons, Hamilton, Illinois.

Jones, Ronald L., and Walter C. Rothenbuhler. 1964. Behavior genetics of nest cleaning in honey bees. II. Responses of two inbred lines to various amounts of cyanide-killed brood. Animal Behaviour (in press).

Lindauer, Martin. 1961. Communication among social bees. Harvard University Press, Cambridge, Massachusetts.

Michener, Charles D., and Mary H. Michener. 1951. American social insects. D. Van Nostrand Company, Inc., New York, N. Y.

Park, O. W. 1936. Disease resistance and American foulbrood. Am. Bee J. 74:12-14.

Ribbands, C. R. 1953. The behaviour and social life of honeybees. Bee Research Association Ltd., London.

Root, J. A. (ed.). 1962. The ABC and XYZ of bee culture. The A. I. Root Company, Medina, Ohio.

Rothenbuhler, Walter C. 1958. Genetics of a behavior difference in honey bees. Proc. 10th Inter Cong. of Genetics, Vol. II:242.

———. 1960. A technique for studying genetics of colony behavior in honey bees. Am. Bee J. 100: 176-198.

———. 1964. Behavior genetics of nest cleaning in honey bees. I. Responses of four inbred lines to disease-killed brood. Animal Behaviour (in press).

Rothenbuhler, Walter C., and Victor C. Thompson. 1955. Resistance to American foulbrood in honey bees. I. Differential survival of larvae of different genetic lines. J. Econ. Entomol. 49:470-475.

Thompson, Victor C. 1964. Behavior genetics of nest cleaning in honey bees. III. Effect of age of adult bees on their response to disease-killed brood. J. Apic. Res. (in press).

Woodrow, A. W., and E. C. Holst. 1942. The mechanism of colony resistance to American foulbrood. J. of Econ. Entomol. 35:327-330.