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Characteristic Field Symptoms Comprising Honeybee Hairless-Black Syndrome Induced in the Laboratory by a Virus

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All the characteristic symptoms comprising hairless-black syndrome found in the field were reproduced in the laboratory by transferring virus-fed adult honeybees, *Apis mellifera,* to cages containing healthy bees. The experimental, virus-fed bees showed high mortality and various combinations of other symptoms, such as paralysis, withdrawal from the cluster, trembling, subjection to attack by healthy bees, and the hairless-black condition. Control bees, treated identically except not fed virus, displayed none of these symptoms. Virus-fed bees transmitted the disease to other bees; control bees did not. Suspensions made from experimental bees contained virus; suspensions made from control bees did not. It is concluded that the virus is the causal agent of hairless-black syndrome.

INTRODUCTION

Hairless-black syndrome in colonies of the honeybee, *Apis mellifera,* includes a variety of symptoms. Sick adult bees may tremble, may become paralyzed, and frequently withdraw from the main cluster to remote parts of the hive, where they die. Some sick bees are attacked by their hivemates and become hairless and black (Kulinčević et al., 1969; Waddington, 1971). Adult bees displaying these symptoms contain a nonoccluded virus (Rinderer, 1974). This virus, fed to bees in laboratory cages, has induced high mortality with concurrent symptoms of trembling, paralysis, and withdrawal. Only occasionally, however, has attacking behavior or the hairless-black condition been seen following administration to caged bees of the virus in sugar syrup. Since attacking behavior is a prominent symptom in apiary colonies, it is considered essential to see it in the laboratory. Until this symptom is seen, a question remains about the relationship of the isolated virus to the disease in the field. Furthermore, hair ingested during attacking behavior contributes to the increased incidence of disease (Rinderer and Rothenbuhler, 1975).

This paper reports the results of two experiments. The first experiment explored the possibility that the virus particles observed by Rinderer (1972) were not the exclusive source of mortality and symptoms. This experiment involved feeding bees various density-gradient fractions of material extracted from bees with hairless-black syndrome.

The second experiment was based upon the possibilities that sick bees do not attack other sick bees and that when given virus in their food, all the bees in a cage become affected. The experiment was designed, therefore, to bring sick bees into contact with healthy ones. The objective was to see whether or not attacks occurred under these conditions and whether or not sick bees became hairless and black. Purified virus was fed during the experiment, and after the experiment, virus was extracted from experimental groups.

MATERIALS AND METHODS

Virus for experimental feeding was harvested by grinding with a mortar and pestle the heads of bees displaying hairless-black syndrome and mixing the resulting slurries with sterile distilled water. These

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suspensions were subjected to two cycles of differential centrifugation utilizing 480, 9750, 9750, and 73 950*g* for 15, 60, 60, and 120 min, respectively. Resulting pellets were resuspended in 0.5 ml of sterile distilled water and layered on 10/40 (w/w) continuous ribonuclease-free sucrose (Sigma Chemical Co., St. Louis, Mo.) density gradients. These gradients were centrifuged at 98 600*g* for 30 min, which produced two bands of material.

In the first experiment, extracted material from 17 sick bees was subjected to centrifugation on a density gradient. Five fractions, identified as the two bands and the adjacent clear volumes above, between, and below them, were collected separately. After collection, each of the five fractions was diluted to 7.5 ml in 50% by volume ribonuclease-free sucrose syrup and fed to five samples of 50 adult bees, aged 0–1 day, contained in laboratory cages described previously (Kulinčević et al., 1973). Feeding continued for 3 days with freshly prepared fractions given daily. Other cages of bees, used as experimental controls, received sucrose syrup with nothing added to it during the 3 days. Throughout the entire experiment, all bees had access to sterile distilled water. After the period when they received experimental treatments, bees had access to ribonuclease-free sucrose syrup. Each cage was inspected daily for 14 days, including the days inoculum was fed, to remove, count, and record dead bees. Data were analyzed by least squares analysis of variance and a least significant difference test.

For the second experiment, 48 cages, each containing 50 commercial-stock bees, were prepared using adult bees aged 0–1 day. The bees in four cages received density-gradient purified virus in ribonuclease-free sucrose syrup once a day over the next 3 days. Total dosage per bee was approximately 3×10^8 virus particles as determined by the latex ball–virus mixture method of Williams and Backus (1949). Four control cages of bees were handled identically except that they were fed ribonuclease-free sucrose syrup with no virus. Both experimental and control bees had access to sterile distilled water. When all bees were 4–5 days of age, virus-fed bees were anesthetized with CO_2, removed from their cage, paint-marked on the dorsal thorax for later recognition, and put by groups of 10 into 20 cages containing non-virus-fed bees of the same age. The four cages of control bees were treated in the same way and put into the remaining 20 cages of healthy bees. These 40 cages were provided with sterile distilled water and ribonuclease-free sucrose syrup. Each of the 40 cages of bees was observed 20 sec for attacking behavior once per day over the next 14 days. Dead bees were counted and removed each day. Data were analyzed by analysis of variance and a least significant difference test.

Suspensions to be examined for virus were prepared from heads of (1) dead bees fed virus and transferred, (2) dead bees not fed virus and transferred, (3) dead bees of the group serving as hosts to bees fed virus, and (4) dead bees of the group serving as hosts to bees not fed virus. These suspensions, stained with 1% neutral phosphotungstate, were examined with a Zeiss EM9-S electron microscope for the presence of virus particles. Estimates of numbers of virus particles were made using the method of Williams and Backus (1949).

RESULTS AND ANALYSIS

Of the fractions from density gradient separation, material in the upper band stood alone in its ability to produce mortality (Table 1). The 46% average mortality caused by this material is significantly higher ($P < 0.01$) than mortalities occurring after treatment with any other fraction. All the other treatments resulted in mortalities not significantly different from each other or from the control. Numerically, the clear fraction above the first band caused higher mortality than found in the controls. This fraction may have contained a small amount of virus. None of the treatments resulted in

TABLE 1
Average Least-Squares Percentage Mortality of Bees Fed Fractions of Density-Gradient Separated Material from Bees with Hairless-Black Syndrome

Fraction fed	Number of replicates[a]	Average percentage of mortality ($\bar{X}$ ± S.D.)
Top	5	13.6 ± 3.6
Upper band	5	45.8 ± 3.6[b]
Middle	5	7.2 ± 3.6
Lower band	5	4.8 ± 3.6
Bottom	4	5.6 ± 4.2
Control	5	3.6 ± 3.6

[a]Bees per replicate = 50.
[b]Significantly different ($P < 0.01$).

observed displays of attacking behavior or hairless-black bees. However, withdrawal behavior, trembling, and paralysis were prominent in samples that received material from the upper band.

Electron microscope observations revealed virus particles such as those described by Rinderer (1974) in the upper band and apparently cellular debris in the lower band.

Although not all the symptoms of hairless-black syndrome were observed in the test samples showing disease, it is concluded that the virus was the exciting agent of the symptoms that were observed.

In the experiment in which virus-fed bees were transferred, all the characteristic field symptoms comprising hairless-black syndrome, including attacking behavior and hairless-black bees, were seen. The bees fed virus, marked, and then transferred showed withdrawal behavior, trembling, and paralysis and had an average mortality of 78% (Table 2). Furthermore, virus-fed bees in 19 of the 20 experimental samples were seen to be attacked by healthy bees a total of 40 times. These observed attacks included two occasions on which a virus-fed bee was attacked simultaneously by two healthy bees. Even though attacks were most prominent during the first 3 days after transfer, they were observed to occur through the 6th day. No attacks were seen in the last 8 days of the experiment. During the 6-day attacking period, certain of the transferred, virus-fed bees became increasingly hairless and 42 of the 156 that died were hairless and black.

The bees serving as hosts to the transferred, virus-fed bees eventually showed certain symptoms of disease. These symptoms, which developed during the middle or latter part of the 14-day observation period, included withdrawal, trembling, paralysis, and mortality averaging 33%. None was observed to be attacked, although

TABLE 2
Mortality, Presence of Virus, Hairless-Black Bees, and Attacking Behavior in Experimental Samples Combining Virus-Fed and Nonvirus-Fed Bees Observed for 14 Days

Makeup of each sample	Number of samples	Average percentage of mortality ($\bar{X}$ ± S.D.)	Presence of virus in dead bees	Percentage of hairless-black dead bees[a]	Times attacks seen in daily 20-sec observations
Experimentals	20				
50 nonvirus fed		33.4 ± 9.3[b]	Yes[c]	3[b]	0
10 virus fed added		78.0 ± 16.7[b]	Yes[c]	27[b]	40[b,d]
Controls	20				
50 nonvirus fed		6.0 ± 8.2	No[e]	0	0
10 nonvirus fed added		5.0 ± 2.1	No[e]	0	0

[a]Calculated from the total dead in each category.
[b]Significantly different in the respective categories ($P < 0.01$).
[c]In all 20 samples.
[d]Average per sample was 2.0 ± 1.8 with 19 of 20 samples showing attacks.
[e]In all samples having mortality. Samples not having mortality were not examined.

11 of the 334 bees that died were black and hairless.

Among the controls, no characteristic symptoms of hairless-black syndrome were evident. Average mortality (6%) in the transferred nonvirus-fed bees was significantly lower ($P < 0.01$) than that occurring in the transferred virus-fed samples. Bees in the transferred control samples were not observed to be attacked, and none of the few bees that died was hairless and black. The bees serving as hosts to the transferred nonvirus-fed bees showed a 5% mortality, significantly lower ($P < 0.01$) than the mortality of the samples hosting the transferred virus-fed bees.

Virus was found only in extracts from the heads of dead bees showing the symptoms comprising hairless-black syndrome. Transferred virus-fed bees that died and the bees hosting them each contained as groups more of the nonoccluded virus than was originally fed. Transferred nonvirus-fed bees and the bees hosting them were not found to contain virus.

It is concluded that bees sick as a result of infection with the virus are sometimes attacked but primarily, or only, by healthy bees. It is further concluded that the virus is the causal agent of hairless-black syndrome.

DISCUSSION

This investigation demonstrates that with an appropriate experiment, the characteristic field symptoms comprising hairless-black syndrome are generated in the laboratory after feeding purified virus. Furthermore, the number of virus particles extracted from the dead experimental bees indicated that virus replication occurred during the experiment. This evidence clearly shows that the virus is the causal agent of hairless-black syndrome.

Beyond this demonstration, the investigation refocuses the question of the relationship between the virus causing hairless-black syndrome and the chronic bee paralysis virus (CBPV) described by Bailey et al. (1963). Horvath and Rothenbuhler (1969) concluded, after consideration of reported gross and histopathological evidence, that the syndrome was different from chronic bee paralysis. One basis for this conclusion was Bailey's (1965) report that he had not observed hairless-black bees. Our investigation shows that attacking behavior, and consequently hairless-black bees, only occurs when healthy bees encounter sick bees. Bailey (1965) did not use an experimental design that would permit such encounters and thus did not see attacking behavior or hairless-black bees in his experiments. Another basis for the conclusion of Horvath and Rothenbuhler was the description of a variety of virus-sized particles found by electron microscopy (Kulinčević et al., 1969). These particles, suspected to be agents of hairless-black syndrome, were not similar to the CBPV described by Bailey et al. (1963). The virus that the present work has shown to be the causative agent of hairless-black syndrome is strikingly similar to the CBPV described by Bailey et al. (1963). Thus, there is the possibility that the two viruses and the two maladies are related. To explore the possible virus relationships, a serological investigation is now under way.

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REFERENCES

BAILEY, L. 1965. Paralysis of the honey bee, *Apis mellifera* Linnaeus. *J. Invertebr. Pathol.*, **7**, 132–140.

BAILEY, L., GIBBS, A. J., AND WOOD, R. D. 1963. Two viruses from adult honey bees (*Apis mellifera* Linnaeus). *Virology*, **21**, 390–395.

HORVATH, R. J., AND ROTHENBUHLER, W. C. 1972. The gross and histological pathology of a hairless-black syndrome in the adult honey bee, *Apis mellifera*. *J. Invertebr. Pathol.*, **20**, 255–263.

KULINČEVIĆ, J. M., ROTHENBUHLER, W. C., AND STAIRS, G. R. 1973. The effect of presence of a queen upon outbreak of a hairless-black syndrome in the honey bee. *J. Invertebr. Pathol.*, **21**, 241–247.

KULINČEVIĆ, J. M., STAIRS, G. R., AND ROTHENBUHLER, W. C. 1969. A disease of the honey bee causing behavioral changes and mortality. *J. Invertebr. Pathol.*, **14**, 13–17.

RINDERER, T. E. 1974. Infectivity degradation by ribonuclease and table sugar of a nonoccluded virus inoculum prepared from the honey bee. *J. Invertebr. Pathol.*, **24**, 120–121.

RINDERER, T. E., AND ROTHENBUHLER, W. C. 1975. The fate and effect of hairs removed from honeybees with hairless-black syndrome. *J. Invertebr. Pathol.* **26**, 305–308.

WADDINGTON, K. D. 1971. Effects of hairless-black disease on honey-bee behavior. Unpublished M.S. thesis, The Ohio State University, Columbus, Ohio.

WILLIAMS, R. C., AND BACKUS, R. L. 1949. Macromolecular weights determined by direct particle counting. I. The weight of the Busby Hunt virus particle. *J. Amer. Chem. Soc.*, **81**, 4052–4057.