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# MODIFICATION OF CHRONIC BEE PARALYSIS IN HONEYBEE COLONIES BY RIBONUCLEASE

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## Summary

Symptoms of chronic bee paralysis (CBP) were monitored in naturally infected small honeybee colonies in flight cages. The colonies were fed sequentially either honey and then ribonuclease-free sucrose (R-FS), or R-FS and then R-FS with added ribonuclease. Each food was administered for 3-4 weeks. Both attacking behaviour and daily mortality were used as indicators of the level of CBP. Honey as treatment 1 resulted in significantly less CBP than did R-FS as treatment 1. Honey as treatment 1 also resulted in significantly less CBP than R-FS as treatment 2. When R-FS was treatment 1, the addition of ribonuclease to R-FS as treatment 2 gave a significant decrease in the level of CBP.

## Introduction

Since 1967 personnel at our laboratory have investigated a condition of the honeybee (*Apis mellifera*) called hairless-black syndrome (Rothenbuhler et al., 1969). A virus was isolated from bees showing this syndrome (Rinderer, 1974) and demonstrated to be the causative agent (Rinderer & Rothenbuhler, 1976). Serological investigations (Rinderer & Green, 1976) have shown that this virus has antigenic specificity indentical to that of the virus causing chronic bee paralysis (CBP). Laboratory infectivity experiments on small groups of honeybees fed a mixture of virus prepared from bees showing hairless-black syndrome, and ribonuclease in sucrose solution, have shown a degradation of infectivity by ribonuclease (Rinderer, 1974). These experiments suggested the possibility that ribonuclease might reduce the level of CBP in naturally infected colonies, and that the level of disease in a colony may be influenced by substances in the natural diet. Our investigation was conducted to explore these possibilities.

## Materials and Methods

Three colonies (801, 804, 807) displaying moderate and approximately equal levels of hairless-black syndrome, from the line of bees selected for susceptibility to the syndrome (Kulinčević & Rothenbuhler, 1975) served as a source of bees. During the summer of 1975 two queenless nuclei were established on the same day from each of these colonies. Each nucleus had approximately 1 kg of adult bees and 2 Hoffman frames containing combs with approximately equal amounts of sealed and unsealed brood, but with very little or no honey or pollen. One of the two nuclei from each source contained a comb of partly sealed honey from the original colony, and the other contained an empty comb. Each nucleus hive was fitted with a dead-bee receptacle, and placed in a flight cage ($2{\cdot}7 \times 2{\cdot}7 \times 5{\cdot}3$ m) to prevent the bees foraging in the field. Cages contained either one nucleus or two which would receive the same treatment.

Nuclei were fed sequentially two foods throughout the period of observation. One nucleus from each source colony had been given honey from the appropriate source

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colony as treatment 1, and then after 19-31 days when all honey was removed, it was fed a solution (50% by volume) of ribonuclease-free sucrose* (R-FS) for 15-27 days as treatment 2. The other nucleus from each source colony was fed R-FS for 20-30 days, and then R-FS to which was added 2000 ppm type X1-A bovine powdered pancreatic ribonuclease* (R-FS + R) for 22-29 days. When the food was changed in each nucleus, brood from the source colony was added, as a continuing source of young bees: 1 or 2 frames, equivalent to 1 Hoffman frame full of sealed and unsealed brood. Throughout the experiments, a liberal supply of bee-collected pollen was provided in a feeding dish inside each nucleus hive.

During the experiments, each nucleus was inspected daily. Dead and paralysed bees in the dead-bee traps were collected and counted, and attacking behaviour characteristic of the disease (Kulinčević et al., 1969; Waddington, 1971) was observed. The numbers of attacks seen during 1-min observation periods, at the hive entrance and at the opened top of the hive, were recorded.

Data on attacking and on mortality for the nuclei from each source colony were analysed as separate experiments, by analysis of variance and least significant difference tests. Attacking data were transformed to $log\ (x + 1)$ in order to achieve uniformly normal distributions for analysis (Snedecor & Cochran, 1967). The numbers of dead and paralysed bees were added together prior to analysis. Mortality data were also submitted to a regression analysis (mortality through time). Since the slopes of mortality curves proved to be similar within treatment groups, they were also analysed by an analysis of variance and a least significant difference test.

## Results and Analyses

Attacking data are presented in Table 1, and mortality data in Table 2. Three comparisons of the average effect of treatments through time can be made. Comparison 1, between treatments 1 and 2 (R-FS and R-FS + R) within each No. 1 nucleus; comparison 2, between treatments 1 and 2 (Honey and R-FS) within each No. 2 nucleus; Comparison 3, between different treatments 1 (R-FS and Honey) of the two nuclei from each source colony. Statistical results are presented in Table 3, and slopes of mortality data are compared in Table 4 and Fig. 1.

Analysis of attacking data (Table 1, Table 3) shows that feeding with R-FS consistently resulted in more attacks than other treatments did. When R-FS preceded R-FS + R (Comparison 1), nuclei from colonies 804 and 807 showed a highly significant ($P < 0{\cdot}01$) reduction in the number of attacks after ribonuclease was added to the food. The nuclei from source colony 801 showed a smaller, nonsignificant, decrease in the number of attacks. When honey preceded R-FS (Comparison 2), R-FS feeding resulted in more attacks in nuclei from all 3 source colonies. When R-FS preceded honey (Comparison 3), a significantly higher ($P < 0{\cdot}01$) number of attacks occurred when R-FS was fed to nuclei from source colonies 804 and 807. Nuclei from colony 801 showed a nonsignificant increase. The analysis of attacking data supports the hypothesis that ribonuclease reduces the level of CBP, and that certain substances—present in at least some honeys—also inhibit the disease. Ribonuclease has not so far been reported in honey.

Comparison of average daily mortalities provides further support for the hypothesis concerning honeys (Table 2, Table 3). In Comparison 3, R-FS generally resulted in higher average daily mortalities (801, higher; 804, significantly higher, $P < 0{\cdot}01$; 807, significantly higher, $P < 0{\cdot}05$). In Comparison 2 (honey as food 1, R-FS as food 2), R-FS resulted in a greater average daily mortality in all 3 nuclei ($P < 0{\cdot}01$).

* Obtained from Sigma Chemical Company, St. Louis, Missouri.

TABLE 1. Average number of attacks (with standard deviations) counted during daily 2-minute observation periods of honeybee nucleus colonies during the indicated feeding periods. Two nuclei from each of the three source colonies.

Ribonuclease-free sucrose was fed as an aqueous solution approximately 50% by volume. Where indicated, ribonuclease was added to the sucrose solution at 2000 ppm. Honey was fed as combs of sealed and unsealed honey from the appropriate source colony. Data were transformed to log $(x+1)$ and analysed by analysis of variance and LSD test for each source colony; see Table 3.

| *Nucleus colony* | *Food sequence* | *Source colony* 801 | 804 | 807 |
|---|---|---|---|---|
| 1 | First feeding period: *ribonuclease-free sucrose* | | | |
| | No. days fed | 20 | 22 | 30 |
| | No. attacks/day | 4·70±3·95 | 8·13±6·49 | 2·12±2·10 |
| | Second feeding period: *ribonuclease-free sucrose + ribonuclease* | | | |
| | No. days fed | 22 | 29 | 27 |
| | No. attacks/day | 1·59±1·22 | 2·58±3·14 | 0·29±0·91 |
| 2 | First feeding period: *honey* | | | |
| | No. days fed | 19 | 24 | 24 |
| | No. attacks/day | 1·40±1·40 | 0·47±0·66 | 1·09±1·51 |
| | Second feeding period: *ribonuclease-free sucrose* | | | |
| | No. days fed | 15 | 29 | 27 |
| | No. attacks/day | 12·26±4·60 | 3·82±3·01 | 6·66±5·12 |

TABLE 2. Average numbers of dead and paralysed honeybees (with standard deviations) collected daily from traps attached to nucleus hives during the indicated feeding periods. Two nuclei were observed from each of the three sources colonies.

Foods and analysis as in Table 1.

| *Nucleus colony* | *Food sequence* | *Source colony* 801 | 804 | 807 |
|---|---|---|---|---|
| 1 | First feeding period: *ribonuclease-free sucrose* | | | |
| | No. days fed | 20 | 22 | 30 |
| | No. bees dead/day | 20·78±19·20 | 10·84±12·46 | 10·02±24·38 |
| | Second feeding period: *ribonuclease-free sucrose + ribonuclease* | | | |
| | No. days fed | 22 | 29 | 27 |
| | No. bees dead/day | 19·10±13·81 | 21·31±11·31 | 11·96± 8·71 |
| 2 | First feeding period: *honey* | | | |
| | No. days fed | 19 | 24 | 31 |
| | No. bees dead/day | 10·25± 9·24 | 1·80± 2·13 | 2·12± 2·52 |
| | Second feeding period: *ribonuclease-free sucrose* | | | |
| | No. days fed | 15 | 29 | 27 |
| | No. bees dead/day | 65·37±33·00 | 17·42±12·42 | 26·23±17·37 |

TABLE 3. Statistical levels of difference obtained from analyses of data presented in Tables 1 and 2 for nuclei from 3 source colonies.

Comparison 1 is between treatments 1 and 2 (R-FS and R-FS + R) within nuclei. Comparison 2 is between treatments 1 and 2 (honey and R-FS) within nuclei. Comparison 3 is between the 2 nuclei from 1 colony, both under treatment 1 (R-FS and honey).

NS = not significant; * = $P<0\cdot05$; ** = $P<0\cdot01$. The treatment listed after the level of significance is the one that resulted in the higher number of attacks, or of dead bees.

| | *Source colony* | | |
|---|---|---|---|
| *Comparison* | 801 | 804 | 807 |
| Comparison 1 | | | |
| No. attacks | NS, R-FS | **, R-FS | **, R-FS |
| No. dead bees | NS, R-FS | *, R-FS + R | NS, R-FS + R |
| Comparison 2 | | | |
| No. attacks | **, R-FS | **, R-FS | **, R-FS |
| No. dead bees | **, R-FS | **, R-FS | **, R-FS |
| Comparison 3 | | | |
| No. attacks | NS, R-FS | **, R-FS | **, R-FS |
| No. dead bees | NS, R-FS | **, R-FS | *, R-FS |

TABLE 4. Slope and (in brackets) intercept, each with standard deviation, of the regression of daily mortality on time for two nuclei from each of the three colony sources. The foods are described in Table 1.

Analysis was by analysis of variance and LSD test: $a$ = significantly different from all other means; $a$*, $P<0\cdot05$; $a$**, $P<0\cdot01$.

| | *Feeding sequence for nucleus* 1 | | *Feeding sequence for nucleus* 2 | |
|---|---|---|---|---|
| *Source colony* | *Sucrose* | *Sucrose + ribonuclease* | *Honey* | *Sucrose* |
| 801 | $1\cdot74\pm0\cdot30$ | $-2\cdot10\pm0\cdot3$ | $0\cdot62\pm0\cdot18$ | $1\cdot29\pm1\cdot38$ |
| | $(-4\cdot58\pm5\cdot03)$ | $(99\cdot03\pm11\cdot77)$ | $(1\cdot21\pm3\cdot04)$ | $(16\cdot27\pm5\cdot32)$ |
| 804 | $1\cdot35\pm0\cdot18$ | $-0\cdot99\pm0\cdot17$ | $0\cdot16\pm0\cdot04$ | $-0\cdot40\pm0\cdot29$ |
| | $(-7\cdot42\pm2\cdot86)$ | $(61\cdot90\pm7\cdot34)$ | $(-0\cdot43\pm0\cdot7)$ | $(33\cdot48\pm12\cdot35)$ |
| 807 | $1\cdot20\pm0\cdot37$ | $-0\cdot63\pm0\cdot14$ | $0\cdot33\pm0\cdot09$ | $0\cdot38\pm0\cdot37$ |
| | $(-11\cdot10\pm7\cdot55)$ | $(43\cdot31\pm7\cdot22)$ | $(-2\cdot68\pm1\cdot91)$ | $(7\cdot50\pm8\cdot15)$ |
| Mean ± SD | $1\cdot43\pm2\cdot27a$* | $-1\cdot24\pm0\cdot76a$** | $0\cdot37\pm0\cdot23$ | $0\cdot42\pm0\cdot84$ |

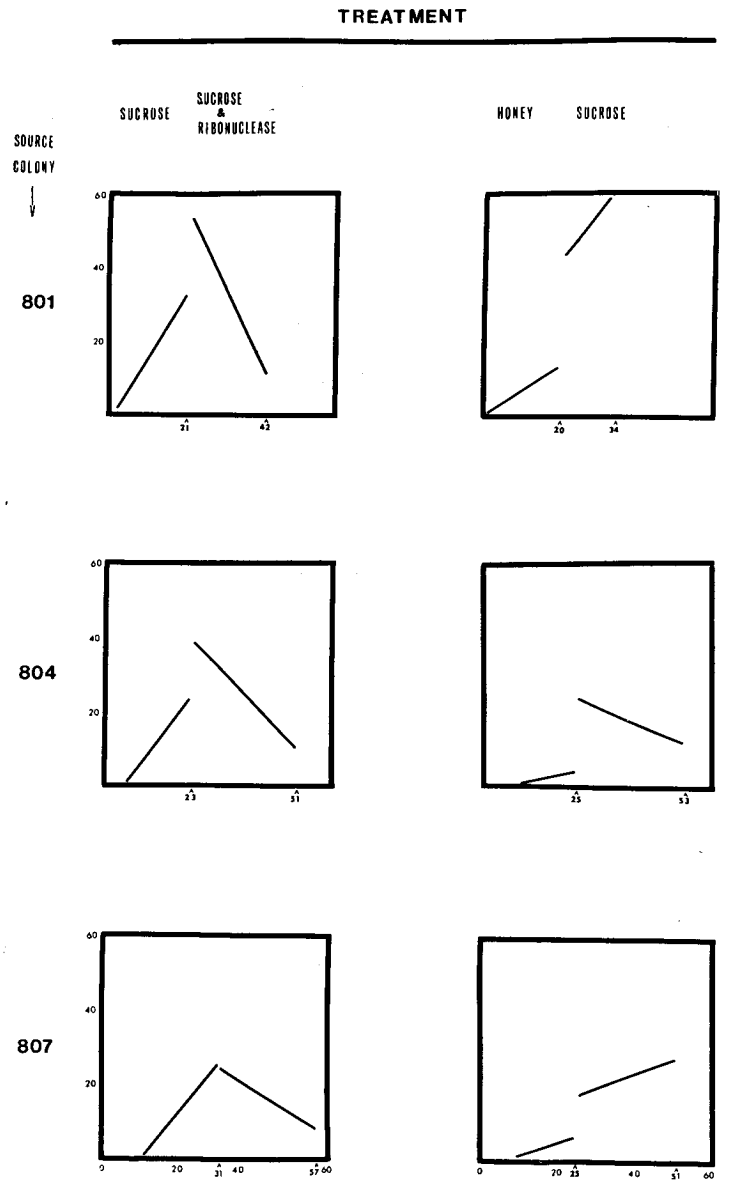


Fig. 1. Graphic presentation of the results in Table 4. Number of paralysed and dead bees (vertical axes) on each day (horizontal axes), up to the final day indicated.

Support for the hypothesis concerning the influence of added ribonuclease does not emerge from a comparison of *average daily mortalities* (Table 2). When R-FS as food 1 in a sequence is compared with R-FS + R as food 2 (Comparison 1), the average daily mortalities for nuclei from two source colonies (801, 807) are quite similar. The data obtained from the nucleus from one source colony (804) show a significantly higher ($P < 0{\cdot}05$) average daily mortality during the period when R-FS + R was being fed.

Support for the hypothesis concerning the influence of ribonuclease does emerge from a comparison of the *regression of mortality on time* (Table 4, Fig. 1). The addition of ribonuclease to the diet of nuclei previously fed only R-FS resulted in the highly significant ($P < 0{\cdot}01$) change, from mortality increasing with time to mortality decreasing with time. This reversal shows that ribonuclease reduced the level of CBP in the nuclei.

Support for the hypothesis concerning honeys also emerges from a comparison of regressions of mortality on time (Table 4, Fig. 1). The slopes for honey as food 1 in the sequence and R-FS as food 2 are rather small, and are not significantly different: there was no substantial change in mortality with time. However, the intercepts of regressions associated with R-FS as food 2 are considerably greater than those for honey. This reflects the greater average daily mortality in nuclei fed R-FS for this comparison. A third comparison, between R-FS as food 1 and honey as food 1, showed a significantly greater ($P < 0{\cdot}05$) average slope of mortality on time for the bees receiving R-FS.

From the evidence of both attacking behaviour and mortality data, it is concluded that ribonuclease reduces the level of CBP. It is further concluded that certain substances present in at least some honeys (perhaps ribonuclease) also inhibit the disease.

## Discussion

Although these were not field experiments, the conditions approximate more closely a natural occurrence of CBP than those in Rinderer's 1974 experiments, with small groups of bees in laboratory cages. Because of the structure of the 1974 experiments, it was not clear what stage in the infection process was disrupted by ribonuclease. The present experiments give indication that the enzyme interferes with the virus before the infectious process results in sick bees. When nuclei containing many sick bees were fed ribonuclease, the reduction in mortality occurred only slowly, and sick bees died in the presence of the enzyme. The reduction in mortality appears to have stemmed from a reduction in the number of bees that were infected when the enzyme was present.

This experiment suggests that a low level of ribonuclease in natural foods of bees is one environmental factor that initiates the onset of an epizootic of CBP. Since the homopterous insects that produce honeydew remove nitrogenous compounds selectively (see e.g. Wigglesworth, 1965), honeydew presumably contains little ribonuclease. This condition may be a factor in the development of the form of CBP known as *Schwarzsucht* (black disease). It may be that flows of "water white" nectar, which some North American beekeepers have observed to be associated with outbreaks of CBP (personal communication to Rinderer), are also low in ribonuclease, and thereby provide conditions favourable to an epizootic.

It will not be possible in the foreseeable future for this laboratory to test the hypothesis that absence of ribonuclease is an important environmental factor in causing epizootics of CBP, and we therefore hope that other scientists will be able to do so.

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