IN VITRO AND *IN VIVO* STUDIES OF *BACILLUS LARVAE*

by

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INTRODUCTION

American foulbrood (AFB) is one of the major brood diseases of the honey bee, *Apis mellifera* L. This disease is incited by the bacterium, *Bacillus larvae* White. American foulbrood is so named because of the foul odor that accompanies the decomposition of infected larvae and pupae. These decaying masses become "ropy" and can be drawn out like a thread. In the final stages, all that remains in the cells of the comb is the dehydrated remnants of the brood, the so-called scales.

Few investigators have studied AFB extensively. A major portion of the present knowledge regarding the etiology of the disease and the cultural characteristics of the pathogen can be attributed to White (1906, 1907 and 1920). Most studies have been directed to effects of the pathogen on the host, rather than effects of the host on the pathogen. Although serial passage through resistant and susceptible hosts has been shown to influence the virulence of plant and animal pathogens, *B. larvae* has not been examined in this manner. One of the objects of the present study, therefore, was to compare the virulence of *B. larvae* after serial passage through resistant and susceptible lines of bees.

Additional problems that have confronted investigators

of AFB are difficulties in cultivation of the pathogen, production of spores *in vitro* and enumeration of the organism for standardization of doses. Methods for enumeration of *B. larvae* have, unfortunately, been limited solely to direct microscopic counts. This method has two serious disadvantages. First, it is impossible to differentiate a viable from a nonviable spore. Secondly, the experimental error is rather high in contrast to the plate count method. Sturtevant (1924) reported that egg yolk medium was too cloudy for plate counts. Plate counts of *B. larvae* would be possible if a satisfactory medium could be prepared. This medium should be such that adequate growth would result from a single viable spore. Since growth rate and degree of bacterial sporulation are interrelated to a certain extent, in sporogenous bacteria, a relatively high degree of sporulation might be obtained on a better culture medium. Spores would be needed for inoculation of bee larvae because Tarr (1937a) demonstrated that *B. larvae* was infective only when in the spore stage. Therefore, a search was conducted for media appropriate for growth (enumeration) and sporulation of *B. larvae*.

No study has been made to compare the virulence of *B. larvae* spores obtained from *in vivo* and *in vitro* sources, therefore, such comparisons were included in the present study. Spores from *in vitro* sources, starting with a single

spore, should be more homogeneous with regard to their phylogeny than spores from larvae dead of AFB.

Although *Bacillus thuringiensis* is not a known pathogen of the honey bee, the indiscriminate use of this bacterium as a microbial insecticide could result in its introduction into honey bee colonies in large quantities. Therefore, the virulence of *B. thuringiensis* for lines of honey bee larvae susceptible and resistant to *B. larvae* was examined to discover whether the use of microbial insecticides might be detrimental to honey bee colonies and to study the relative effects posed when an insect bred for resistance to one bacterial pathogen is challenged with a different bacterial species.

LITERATURE REVIEW

American foulbrood (AFB) is the name given to a brood disease of the honey bee, *Apis mellifera* L. This disease is caused by *Bacillus larvae* White. The name American foulbrood was given to this malady by White (1906) to distinguish the disease from European foulbrood first studied in Europe. European foulbrood, like AFB, is a larval disease. In the former disease, however, *Streptococcus pluton* and *Bacterium eurydice* have been identified as the causative agents (Bailey, 1960).

Bacillus larvae

Bacillus brandenburgiensis Maassen and *Bacillus burrii* Burri are both synonyms of *Bacillus larvae* White (1920). *Bacillus larvae* is a Gram-positive spore forming bacillus. The vegetative cell measures from 2.5 to 5.0 microns in length and about 0.5 microns in width. The spore measures 0.6 to 1.3 microns (White, 1920). Gelatin is not liquified unless carrot extract is present. Starch is not hydrolyzed by this organism; however, growth and sporulation are enhanced by the presence of soluble starch. No indole is produced by *B. larvae*. Acid with curdling in milk and nitrate reduction are characteristic of *B. larvae* (Breed *et*

al., 1957). Thus, the milk curd test (Holst, 1946) and nitrate reduction test (Lochhead, 1937) have both been used successfully for the diagnosis of AFB.

Cultivation of *Bacillus larvae*

American foulbrood produces characteristic symptoms in larvae and pupae of the honey bee. Larvae and pupae that are normally white in color become brownish when the disease is initiated. As the disease progresses, the insect becomes "ropy". At this stage a toothpick, when inserted into the decaying mass and withdrawn, pulls the material threadlike. Finally, after a month or more, all that remains of the infected larva or pupa is a dry mass referred to as the scale (White, 1920). When aqueous suspensions of these scales are heated to 100° centigrade for one or two minutes and then streaked on an appropriate medium, pure cultures of *B. larvae* are obtained (White, 1906).

The problem of obtaining growth of *B. larvae in vitro* becomes apparent when one reviews the early studies. White (1906) first prepared a bee-larvae agar containing an infusion comprised of one part of healthy brood to two parts of water. Later, White (1920) devised a brood-filtrate agar which required crushing of honey bee brood, extraction of the crushed brood and filter sterilization of the brood

extract. The brood-filtrate agar was not satisfactory for spore germination; thus, White used either egg-yolk suspension or bee-larvae agar for germination of spores.

Lochhead (1928) cultivated _B. larvae_ in a yeast-carrot medium. This medium was prepared with carrots, pressed yeast cells, peptone and K_2HPO_4. Growth of _B. larvae_ was obtained from a smaller inoculum when 0.3 per cent agar was added to the yeast-carrot medium (Lochhead, 1933). The organism grew three to four millimeters below the surface of the latter medium. Substitution of turnip extract for carrot extract resulted in more abundant growth (Lochhead, 1937).

Sturtevant (1932) found that at least 50,000 spores were needed to produce growth on an egg yolk-yeast-carrot extract-peptone medium. However, Tarr (1937b) was able to obtain growth with an inoculum less than 1,000 spores of _B. larvae_ on minced chick embryo in Tyrode solution. Tarr (1938a) later found that beef digest hinders germination of _B. larvae_ spores in embryo brei medium.

Holst and Sturtevant (1940) proposed two new media that were essentially modifications of Lochhead's (1928) medium. Whereas Lochhead used peptone, Holst and Sturtevant used neopeptone. The latter investigators reported that growth of _B. larvae_ could be observed within four days, even when a single spore served as inoculum. Since carrot extract

suppressed sporulation, this component was omitted from the second medium devised for stock culturing and spore production. Later, Lochhead (1942) found that vegetable extract, yeast and egg yolk could be replaced with 100 gammas of thiamine per liter of medium.

Gochnauer (1958) used liver infusion nutrient agar or brain heart liver infusion broth for the isolation of bacteriophage from B. larvae. The latest medium proposed for the cultivation of B. larvae was that of Bailey and Lee (1962). They were able to obtain germination and growth of B. larvae from a small inoculum in a medium containing yeast extract, glucose, KH_2PO_4 and soluble starch.

The problem of obtaining spores in vitro has been somewhat difficult. Sporulation can occur in a number of media, but there seems to be no agreement as to which is the best medium. This could well be due to differences in the strains of B. larvae used by various investigators. Tarr (1937a) was able to obtain spores with his brood filtrate medium. However, he reported that B. larvae lost its ability to form spores when maintained on laboratory media.

Smith et al. (1949) were unable to obtain high spore yields using the medium of Holst and Sturtevant (1940). However, Smith et al. (1949) were able to increase sporulation of B. larvae by 10-50% when pollen extract was added to

their basal medium containing neopeptone, yeast extract, K_2HPO_4 and agar. They found that carrot extract was not effective for sporulation of B. larvae. Foster (1956) reported that Smith et al. (1949) were not able to repeat their experiments. However, Bailey and Lee (1962) reported that aqueous extracts of pollen, freshly collected by bees, increased the ratio of spores to vegetative forms from 1:1 to 2:1.

Foster et al. (1950) reported that they obtained up to 80% sporulation of B. larvae in soy hydrolysate plus Wilson liver B after pre-treatment of the medium with activated charcoal or addition of soluble starch to the medium. The net effect was to increase growth and remove or neutralize naturally occurring antisporulation factors.

Inoculation of Honey Bee Larvae

Disease in healthy colonies has been artificially induced by many methods. White (1907) infected colonies by feeding a mixture of spores of B. larvae in syrup or honey to the colonies. Park (1936) infected brood by inserting a piece of comb containing AFB diseased material into a comb of healthy brood. Spraying combs of brood with aqueous spore suspensions of B. larvae was also successful in inducing AFB (Tarr, 1938b). The latest technique developed

for the inoculation of honey bee brood is the individual inoculation method used successfully by Woodrow and Holst (1942) and subsequently by many investigators, including Rothenbuhler and Thompson (1956).

Tarr (1938b) was not successful in producing AFB in larvae by individual inoculation even when each larva was fed up to 4.7×10^6 spores of *B. larvae*. On the other hand, Woodrow (1942) found that a theoretical concentration of 0.1 spore of *B. larvae* per larva was sufficient to incite AFB. His data showed a wide variation in per cent mortality. With five spores per larva Woodrow (1942) obtained from 37.5 to 100.0 per cent mortality in different combs. Thus, he concluded that only one spore of *B. larvae* was necessary to cause AFB. On the other hand, Kitaoka *et al*. (1959) found that ten spores of *B. larvae* were sufficient to cause AFB in larvae one day after hatching. They found considerable variation in mortality of larvae and were unable to show any dependence of spore numbers on larval removal and mortality.

Alteration of Virulence

Dubos (1950), in writing the biography of Pasteur, describes the methods Pasteur used to alter the virulence of pathogens. Pasteur was able to increase the virulence of rabies for rabbits by serial passage through the brains of

rabbits. Serial passage of the rabies virus in monkeys resulted in a virus that was less virulent to dogs, rabbits and guinea pigs. Cowpox, according to Smith (1934), is smallpox that has been greatly attenuated. It can be seen from these examples that virulence, at least of viruses, can be altered by animal passage.

Virulence is the result of complex interactions of host and pathogen. Lincoln et al. (1946) states that disease is a function of the genetic constitution of the host, the genetic constitution of the pathogen, the dosage of the pathogen, the route of inoculation and the influence of the environment during the production of the pathogen. These workers found that B. anthracis spores produced in a medium containing corn steep liquor showed a reduction in virulence compared with spores from a medium without corn steep liquor. They observed that virulence of B. anthracis could be regained by producing spores on a medium without corn steep liquor. Schneider et al. (1963) reported that differences in cultivation method influenced spore size and susceptibility to radiation. They observed that spores of Clostridium botulinum 51B grown in dialysis sacs were larger than spores obtained in flasks. The larger spores were also found to be more susceptible to radiation.

Felty and Bloomfield (1924) observed that four day old cultures of Streptococcus haemolyticus (Streptococcus

pyogenes) were less virulent than five hour cultures. On the other hand, Wilson (1926) found no difference in virulence during the first week of growth of *B. aertrycke* (*Salmonella typhimurium*). Virulent isolates of *Erwinia amylovora* remained so even after four years in culture media (Ark, 1937).

It is a common occurrence for pathogenic bacteria to become attenuated when grown *in vitro* (McNew, 1938). He found that the virulent strains of *Phytomonas stewarti* (*Xanthomonas stewartii*) were capable of utilizing inorganic nitrogen but the avirulent forms lost this ability.

Ark (1937) in studying *Erwinia amylovora* found that the smooth colony types were virulent as opposed to the rough avirulent types. Examples in the literature regarding this phase dissociation are numerous; usually the smooth forms are virulent and the rough forms are avirulent. Smith *et al.* (1953) found that *B. anthracis* cells from *in vivo* sources were more virulent for guinea pigs than those from *in vitro* sources. This difference was attributed to the increased susceptibility of the *in vitro* cells to phagocytosis. On the other hand, the majority of selected biochemical mutants of *Bacterium typhosum* (*Salmonella typhosa*) were found to retain their full virulence (Bacon *et al.*, 1950). Some mutants showed only a slight loss in virulence with no accompanying change in colony form or morphology.

Virulence has also been altered by serial passage through various animals. Burgess (1930) found that the virulence of Bacillus pestis (Pasteurella pestis) was reduced or abolished by passage through immune or partially immune rats. Likewise, the attenuated cultures could be made virulent by successive passages through normal rats. Zelle (1942) reported that the virulence of Salmonella typhimurium was increased by passage through either resistant or susceptible hosts. The same result was obtained when Shigella gallinarum was inoculated into resistant and susceptible chicks; this was due to the difficulty in survival of the avirulent pathogen (Gowan, 1945). He also found that increases in virulence were sudden and that those isolates with increased virulence were stable with regard to their virulence.

Wellhausen (1937) and Lincoln (1940) both concur that serial passage of Phytomonas stewarti (Xanthomonas stewartii) alters the virulence of the pathogen. Both found that serial passage of X. stewartii in susceptible lines of maize decreases virulence, whereas in resistant lines the virulence is increased. Wellhausen (1937) observed that changes in virulence were possible only within a range. In other words, there were both maximum and minimum levels of virulence.

Virulence of *Bacillus thuringiensis* to Honey Bees

Lecomte and Martouret (1959) reported that no adverse effect was noted on feeding *Bacillus thuringiensis* spores to to adult honey bees. Wilson (1962) noted no disease or reduction in honey production after administering commercial preparations of *B. thuringiensis* spores to honey bee colonies. On the basis of these two studies, it is generally assumed that *B. thuringiensis* is not a pathogen of the honey bee.

MATERIALS AND METHODS

Cultures of Bacillus larvae were obtained from American foulbrood (AFB) diseased material (scales). These cultures were isolated by streaking aqueous suspensions of scales on Trypticase Soy Agar[1]. The isolates were maintained on a medium suggested by Bailey[2]. The medium (medium B) had the following composition:

Yeast extract[3]	1.0 per cent
Soluble starch	1.0 per cent
Agar	0.2 per cent
KH_2PO_4, 0.01 M, pH 6.6	

For a solidified medium (medium BA), the concentration of agar was increased to 1.5%.

Viable counts of B. larvae were made by a ten tube MPN method proposed by Halvorson and Moeglein (1940). Plate counts were made by the pour plate technique. Plates were incubated at 37° centigrade for 48 hours. Before counting, two milliliters of a 0.1 per cent aqueous solution of 2,3,5 - triphenyl - 2H - tetrazolium chloride was poured on the surface of the agar, the plate was rocked gently, then the excess indicator solution was decanted. The plates were

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[1]Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland.

[2]Bailey, L., Rothamsted Experimental Station, Harpenden, Hertfordshire, England. Medium for cultivation of Bacillus larvae White. Private communication. 1960.

[3]Difco Laboratories, Inc., Detroit 1, Michigan.

allowed to incubate at room temperature for three to five hours and then were counted (Solberg and Proctor, 1960).

Screening of media was conducted using approximately four milliliters of broth in 13 x 120 millimeter tubes. Each tube was inoculated with a drop (approximately 1,000 organisms) of a 48 hour culture grown at 37° centigrade in medium B, without any agar, using a dropping bottle. Tubes were incubated at 37° centigrade and observations were made every 24 hours for a period of three days. The compositions of media that were screened will be shown in the Results and Discussion section.

In vitro spores of *B. larvae* (Figure 5) were prepared by inoculating 900 milliliter screw cap bottles containing 100 milliliters of medium BA with three milliliters of a 48 hour culture in medium B that had been incubated at 37° centigrade. After one week of incubation at 37° centigrade, the spores were washed from the medium with ten milliliters of sterile distilled water. The spores were then washed twice with sterile distilled water, heat-shocked at 60° centigrade for 30 minutes, the dosage was standardized and the spore suspension was divided into aliquots in small vials. These vials containing the spore suspensions were frozen and stored at -20° centigrade.

The direct microscopic count method of Breed and Brew (1916) was used to determine spore numbers. An A. O.

Spencer phase microscope was used for counting. The use of phase microscopy rather than light microscopy was selected because, in phase microscopy, the possibility of spore removal from the smear during staining and washing is eliminated. Two smears were made by placing 0.01 milliliter of spore suspension on a one square centimeter demarcated area. Twenty-five fields of 0.01 square millimeter each were counted from each smear. The results of the fifty fields (twenty-five fields from each smear) were pooled to obtain the average number of spores per field. This average was then used to calculate the number of spores in the original suspension.

In vivo spores of B. larvae (Figure 6) were prepared as powders by grinding remains of larvae and pupae that had succumbed to AFB. The Sturtevant spore powder was supplied by the late A. P. Sturtevant of Laramie, Wyoming. This spore powder was at least ten years old. The Ames spore powder was prepared in 1959 from scales collected at the Iowa State University Apicultural Laboratory. Both spore powders were kept in a dessicating bottle at room temperature. Spore suspensions were prepared by adding distilled water, heat-shocking at 60° centigrade for 30 minutes and finally standardizing the dosage by use of direct microscopic counts. The spore suspension was then frozen and stored at -20° centigrade. Individual vials were thawed as needed.

Lines of bees have been bred at the Iowa State University Apicultural Laboratory for their susceptibility and resistance to AFB. The Brown resistant and the Van Scoy susceptible lines were used throughout this study for brood sources. The history of these lines is given by Bamrick (1960).

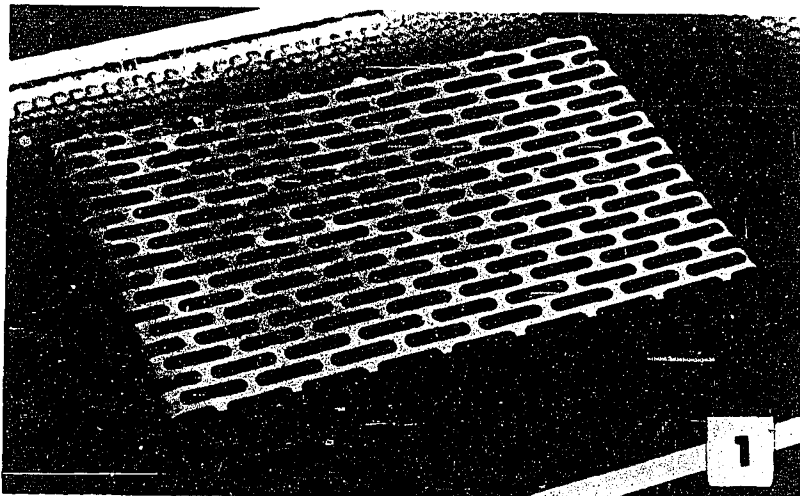
Individual queens were caged on dark test combs in which brood had been previously reared. The five by seven inch queen cages were made with openings to allow worker bees to enter and leave freely, but the exit of the queen was prevented (Figure 1). To obtain larvae of relatively uniform ages, queens were caged for six hour periods. All eggs were assumed to hatch 72 hours from the midpoint of the caging period. At this time, the larvae were assigned an age of zero hour. The ages of brood used in this study were averages: 16 hour larvae were actually 13-19 hours old, 24 hour larvae were 21-27 hours old and 32 hour larvae were 29-35 hours old.

Test combs containing eggs were transferred to a nurse colony prepared from a strong colony of commercial hybrid bees. The colony was prepared by placing a queen excluder between the first and second story, thereby restricting the queen to the first story and preventing further egg laying in the test combs. To insure proper care of the test combs, they were surrounded with capped

Figure 1. A 5" x 7" queen cage

Figure 2. Micrometer syringe used for feeding spores to honey bee larvae

Figure 3. Template used for numbering rows and treatments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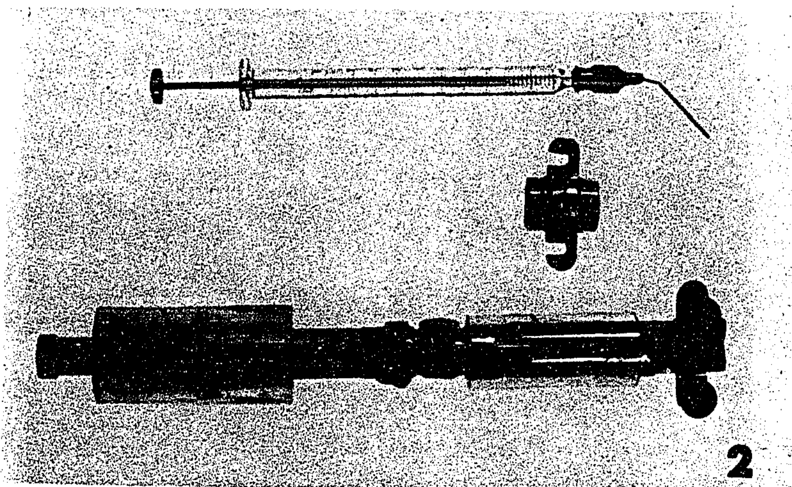
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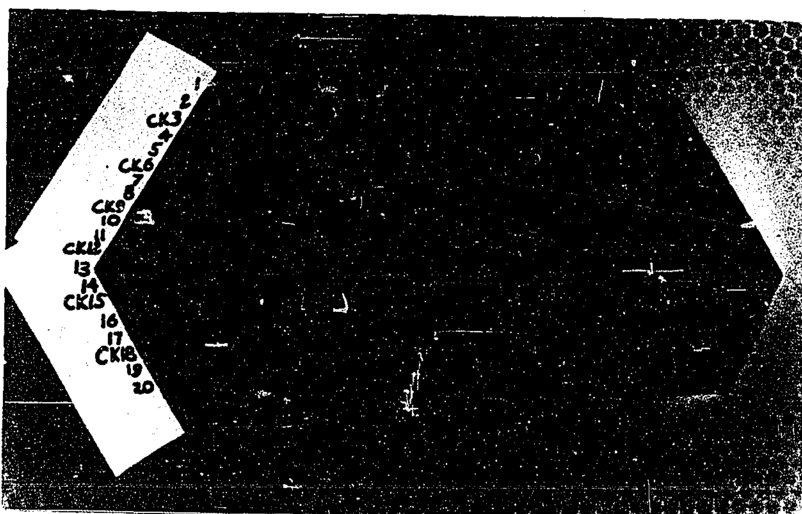
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CK18
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brood from the first story of the nurse colony or from a colony of similar parentage.

After appropriate incubation periods, combs containing the test larvae were brought into the laboratory in a comb carrier. The most densely populated areas were selected for the tests. Templates were made from plastic sheets to denote rows and treatments (Figure 3). Three treatments were utilized on each comb: two rows of spore treatments to one row of distilled water used as a control. Individual inoculations were made with a specially adapted "Agla" micrometer syringe[1]. A 0.1 milliliter Hamilton syringe #710[2] was used instead of the "Agla" syringe (Figure 2). The micrometer was adapted with a plastic barrel having an indentation for each one-third revolution which was calculated to deliver 0.287 cubic millimeter. The syringes were sterilized in the autoclave after each use.

After administering the treatments to the larvae, the combs were returned to their former position in the nurse colony. Twenty-four hours later, a base count was made using the same template used for inoculation to determine the number of larvae remaining in the combs. This count was made to eliminate those larvae which may have been

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[1]"Agla" micrometer syringe - Burroughs Wellcome & Co., Inc., Tuckahoe, N.Y.

[2]Hamilton syringe - Hamilton Company, Inc., Whittier, California.

injured or killed by handling and during inoculations. All calculations, except where noted, were based on the 24 hour base counts. A final count was made 15 days after the eggs hatched to record the number of larvae and pupae dead or missing from the test combs. All individuals missing from the combs were assumed to have died from AFB. In instances where the larvae or pupae died from causes other than AFB, the individuals were subtracted from the base counts.

One *in vitro* *B. larvae* spore suspension, "X", was prepared by making a single colony isolate from AFB scales obtained at the Iowa State University Apicultural Laboratory. Sixteen hour Brown line larvae were fed a dose of 15,000 spores. Later, four more *in vitro* isolates were made: two from the Sturtevant spore powder (1a and 2b) and two from the Ames spore powder (1c and 2c). Five-hundred spores per larva were fed to 24 hour Van Scoy line larvae.

To study the effect of serial passage, a series of three passages was made through honey bee larvae. Bamrick[1] graciously supplied F_1 larvae (Brown x Van Scoy) dead after feeding 15,000 spores per larva of the Sturtevant spores. Spores (SF) were harvested by placing about 20 larvae in a test tube and macerating in distilled water with a glass rod. The mixture was strained through two layers of cheese cloth

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[1]Bamrick, J. F., present adress: Loras College, Dubuque, Iowa.

and washed twice by centrifugation with distilled water. The spores were then heat-shocked at 60° centigrade for 30 minutes, counted and the dosage was standardized so that each larva would be fed 15,000 spores. These spores were fed to both 16 hour Brown and 24 hour Van Scoy line larvae. Spores were harvested by the method described previously for the SF spores.

Spores from the AFB dead 16 hour Brown line larvae were designated SFB and those from the 24 hour Van Scoy line larvae were designated SFV. These spore suspensions were frozen until the following year when they were fed to 16 and 24 hour larvae of the Brown and Van Scoy line larvae. Resulting spores from AFB dead bees of the 16 hour Brown (SFBB) and 24 hour Van Scoy (SFVV) lines were again harvested. The change in virulence due to serial passage in the Brown or Van Scoy line larvae was determined by feeding 500 spores to 16 and 24 hour Brown line larvae. The virulence of SFVV, SFBB and the Sturtevant parental spore powder was compared by feeding 15,000 spores to both 16 and 24 hour Brown and Van Scoy line larvae.

The next series of passages was conducted to determine the influence of larval age on the virulence of B. larvae obtained from the AFB dead brood. Fifteen-thousand Sturtevant spores were fed to both six and 40 hour Van Scoy larvae. To prevent the removal of diseased larvae by the

nurse bees, the test combs were placed in a 30° centigrade incubator as soon as the brood was capped. Spores from AFB dead larvae inoculated at six hours of age were designated SY-1 and spores from AFB dead larvae inoculated at 40 hours of age were designated SO-1. These spore suspensions were washed, heat-shocked, counted and the dosage was standardized so that each larva would be fed 15,000 spores. The SY-1 spores were fed to six hour Van Scoy larvae and the SO-1 to 40 hour Van Scoy larvae. Spore suspensions were then made from the SY-1 inoculated larvae: spores from larvae dead of AFB (SY-2L) and spores from pupae dead of AFB (SY-2P). The same scheme was followed for larvae inoculated with SO-1: spores from larvae dead of AFB (SO-2L) and spores from pupae dead of AFB (SO-2P). The virulence of the spore suspensions SY-2L, SY-2P, SO-2L, SO-2P and the parent Sturtevant spore powder was tested in 24 hour Van Scoy line larvae at the level of 500 spores per larva.

In order to determine whether one passage was enough to alter virulence, both the Sturtevant and Ames spores were fed to 24 hour Van Scoy larvae. The dosage employed for this passage was 15,000 spores. The spore suspension from larvae dead of AFB after feeding Ames spores was designated AV and spores from larvae fed Sturtevant spores were designated SV. The virulence of spore suspensions AV and SV were both compared to their parental spore powders at the level of 500

spores per larva.

Bacillus thuringiensis

A spore suspension of *Bacillus thuringiensis* ATCC 10792 (Figure 7) was prepared by culturing the bacterium on Trypticase Soy Agar for one week at 37° centigrade. Spore suspensions were prepared as described earlier for *B. larvae*, except the dosage was standardized by the use of plate counts.

Both Brown and Van Scoy line queens were caged to obtain larvae that were 16, 24 and 32 hours of age. Each larva received 1,800 spores of *B. thuringiensis*, *B. larvae* (Ames spore powder) or an equal volume of distilled water. The Ames strain of *B. larvae* was included in this experiment for comparative purposes.

RESULTS AND DISCUSSION

In Vitro Studies of *Bacillus larvae*

An attempt was made to discover a simple medium from which further studies on growth and sporulation of *Bacillus larvae* could depart. Many complex media have been used for this purpose; however, constituents such as brood extract, egg-yolk, carrot juice, turnip juice and pollen are not amenable to routine use. The following media were selected for preliminary examination either because they had been used by previous investigators in studies on *B. larvae*, or because they were complex media that were available in dehydrated form: Holst's medium (1940), Lochhead's medium (1942), Brain Liver Heart Infusion Broth[1] (Gochnauer, 1958), Tomato Juice Agar[2], Trypticase Soy Agar[2], and Nutrient Agar[1]. A spore suspension of *B. larvae*, strain "C" prepared from scales, was streaked on each medium, the plates were incubated at 37° centigrade for 72 hours and the number and size of resulting colonies were observed. All media tested were found to support only a limited amount of growth of the test organism. Therefore, *in vitro* studies of *B. larvae*

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[1]Difco Laboratories, Inc., Detroit 1, Michigan.

[2]Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland.

were directed towards examination of environmental conditions with the thought that work on the composition of the growth medium could be resumed when more was known about environmental parameters.

Lochhead's medium (1942) was used to determine the pH range over which B. larvae could grow. This medium was selected because it was the first one in which vegetable extracts were replaced with thiamine. Furthermore, growth on this medium was as good or better than on the others tested thus far. The basal medium had the following composition: glucose, 1.0 gram; K_2HPO_4, 1.0 gram; KNO_3, 0.5 gram; $MgSO_4$, 0.2 gram; $CaCl_2$, 0.1 gram; NaCl, 0.1 gram; and $FeCl_3$, 0.01 gram. The ingredients were suspended in 1.0 liter of distilled water, this basal was boiled and filtered, then 5.0 grams of Bacto-Peptone[1] and 100 gammas of thiamine were added. Aliquots of the medium were adjusted to the appropriate pH values with 0.1N NaOH or HCl prior to sterilization. Tubes of sterile medium were inoculated with two drops of a suspension made from a 48 hour culture of B. larvae, strain "C" grown on Trypticase Soy Agar. The tubes were incubated at 37° centigrade and were examined for growth at 24, 48, and 72 hours (Table 1).

Growth of B. larvae occurred over a range of pH

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[1]Difco Laboratories, Inc., Detroit 1, Michigan.

Table 1. Effect of pH on growth of *B. larvae*

pH	Days of incubation		
	1	2	3
5.0	5[a]	5	5
5.5	5	5	5
6.0	5	5	5
7.0	5	5	5
7.5	5	5	5
8.0	0	1	3

[a]Number of tubes showing growth out of five.

5.0 - 8.0 (Table 1). On the basis of optical density, the optimum was found to lie between pH 6.5 - 7.5; therefore, the pH of the medium was not a critical factor in the cultivation of *B. larvae*.

Although growth of *B. larvae* could be obtained on Lochhead's medium (1942), the quantity of growth obtained was small. At this time, Bailey[1] suggested the use of medium B, the composition of which is shown in the section on Materials and Methods. This medium was simple to prepare and the amount of growth of *B. larvae* was greater on medium

[1]Bailey, L., Rothamsted Experimental Station, Harpenden, Hertfordshire, England. Medium for cultivation of *Bacillus larvae* White. Private communication. 1960.

B than on Lochhead's medium.

Incubation temperatures permitting growth in medium B without any agar were also examined. Duplicate flasks were inoculated with about 100,000 cells of a 48 hour culture of _B. larvae_ strain "C" in medium B without agar and incubated at 31, 34, 37, and 40° centigrade. Growth was present in all flasks after 24 hours of incubation; however, in 48 hours cell lysis was evident in flasks incubated at 40° centigrade. Since no difference in quantity of growth could be found in the other temperatures, 37° centigrade was selected for all the _in vitro_ studies.

Definite regions of growth can be noted in medium B containing various levels of agar. The agar concentrations in per cent, represented in Figure 4, are, from left to right 0.50, 0.20, 0.10, 0.05 and 0.00. Without agar, the cells settle to the bottom. As the agar concentration is increased, thus prohibiting the settling of cells and limiting oxygen penetration into the medium, the area of turbidity becomes restricted to the upper regions of the tube. This growth behavior of _B. larvae_ is in conformity with the observations of Lochhead (1928), who classified _B. larvae_ as a facultative anaerobe. Thus, the presence of agar in small concentrations can aid in the detection of most contaminants in cultures of _B. larvae_. Most contaminants are either strict aerobes or facultative aerobes; therefore, they grow on the

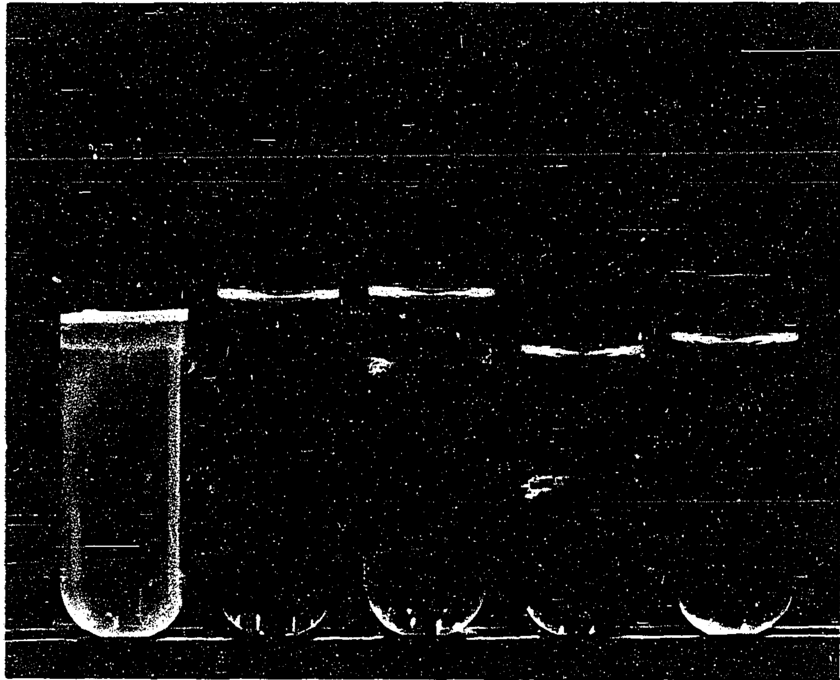

Figure 4. Effect of agar concentrations on growth of *B. larvae*

surface or throughout the medium.

Due to the shortcomings of direct microscope counts, an attempt was made to determine the feasibility of the Most Probable Numbers method of enumeration. A ten tube method (Halvorson and Moeglein, 1940) was used for this purpose, and the data obtained were converted to most probable numbers using a Most Probable Numbers table kindly supplied by Beers[1]. Medium B was inoculated with an *in vitro* spore preparation, "X", that had been standardized using a direct microscopic count.

Even after 50 days of incubation, only 6.1 per cent of the spores germinated to produce visible growth (Table 2).

Table 2. Direct microscopic counts versus Most Probable Numbers method for the enumeration of *B. larvae*

Day of incubation	Spores per milliliter	Per cent germination[a]
0	$1.8x10^{9}$[b]	---
16	$8.5x10^{7}$	4.7
30	$8.8x10^{7}$	4.9
50	$1.1x10^{8}$	6.1

[a]Germination based on resultant growth.

[b]Direct microscopic count.

[1]Beers, R. J., Iowa State University, Ames, Iowa. Most Probable Numbers Table. Private communication. 1960.

The Most Probable Numbers method, therefore, was unsatisfactory in that results would require an incubation period that would be too long to be practical. Furthermore, the medium became dehydrated when incubated for long periods and the concentration of ingredients no longer was the same as when the tubes were first inoculated. It is the opinion of this investigator that the per cent germination of the same spore preparation was greater and the germination occurred in a much shorter time in the honey bee larvae than in the culture medium. Therefore, the limitation of this method seems to be in the medium.

The first component of medium B that was examined in detail was the concentration of yeast extract[1]. Basal medium was prepared by using 1.00 per cent soluble starch and 0.01M KH_2PO_4 at pH 6.6. The results of two experiments, which were pooled, are shown in Table 3. Each tube was inoculated with 1,000 spores of *B. larvae* strain "X".

A concentration 0.5 per cent yeast extract resulted in the greatest number of positive tubes in 48 hours (Table 3). However, over a period of one week incubation, the maximum number of positive tubes contained concentrations of 1.5 and 1.0 per cent of yeast extract. Concentrations of 2.0 per cent and 0.1 per cent yeast extract were unsatisfactory for the growth of *B. larvae*. On this basis, 1.0 per cent

[1]Difco Laboratories, Inc., Detroit 1, Michigan.

Table 3. Effect of level of yeast extract in medium B on growth of _B. larvae_

Per cent yeast extract	Days of incubation 1	2	3	5	7
2.0	0[a]	0	0	5	6
1.5	0	0	5	9	10
1.0	0	3	7	9	10
0.5	0	7	9	9	9
0.1	0	0	0	0	4

[a]Number of tubes showing growth out of ten.

yeast extract was selected as the optimum level.

Media B and BA both contain 0.01M KH_2PO_4. It was felt that the effect of K^+ on germination and outgrowth of _B. larvae_ spores should be investigated because the level might be either toxic or too low to result in any beneficial effect. Media were prepared incorporating KH_2PO_4, K_2HPO_4, and Na_2HPO_4 at concentrations of 0.00, 0.02, and 0.10M. The basal medium consisted of 1.0 per cent each of yeast extract[1] and soluble starch. Concentrations of phosphate in the test media are given in Table 4. All media were adjusted to pH 6.6 with 0.1N KOH and 0.1N HCl before dispensing 25 milliliters of medium per 125 milliliter Erlenmeyer flask. The

[1]Difco Laboratories, Inc., Detroit 1, Michigan.

Table 4. Effect of K^+ and Na^+ ions on growth[a] of *B. larvae*

Medium	Buffer	Molar solution	Days of incubation 1		2	
			Rep.[b] A	Rep. B	Rep. A	Rep. B
1	Na_2HPO_4	0.02	+	+	+++	+++
2	"	0.10	+	+	+	+
3	K_2HPO_4	0.02	-	+	+++	+++
4	"	0.10	-	±	++	++
5	KH_2PO_4	0.02	+	-	++	++
6	"	0.10	-	-	+	+
7	None	--	-	-	++	++

[a]Quantity of growth: (-) none, (±) doubtful, (+) slight to (+++) good.

[b]Replicate.

flasks were sterilized in the autoclave, then 10,000 spores of strain "C" were inoculated into each flask.

A substantial quantity of growth was obtained in medium 7 in the absence of buffer. Less growth of *B. larvae* was apparent in media containing 0.1M KH_2PO_4, K_2HPO_4, or Na_2HPO_4 than when these phosphates were present at the 0.02M level (Table 4). The maximum quantity of growth was observed in media 1 and 3 which contained 0.02M Na_2HPO_4 or 0.02M K_2HPO_4, respectively. Thus, 0.02M Na_2HPO_4 or 0.02M K_2HPO_4

could be substituted for KH_2PO_4 in medium B. Since growth of *B. larvae* occurred without the presence of any added buffer (medium 7), the presence of any of the tested buffers is not necessary for growth.

The effects of Na^+, K^+, and PO_4^{-3} ions on growth of *B. larvae* were examined further using a basal medium composed of 1.0 per cent each of yeast extract[1], soluble starch, and glucose. The addition of glucose was a modification of medium B suggested by Bailey and Lee (1962). Various salts were added to the basal medium in the concentrations listed in Table 5. The media were adjusted to pH 6.6 and each was dispensed into twenty tubes. Following sterilization, the tubes were inoculated with about 1000 vegetative cells of *B. larvae* of either the Ames or Sturtevant strains. As shown in Table 5, no added salts were necessary for the growth of *B. larvae*, although growth appeared to be slightly more rapid in the presence of 0.01M KCl. As in the previous experiment (Table 4), the 0.1M concentrations of Na_2HPO_4 and KH_2PO_4 delayed the growth of *B. larvae*. From the results of these two experiments, it was apparent that neither the cations K^+ and Na^+ nor the anion PO_4^{-3} were essential for the growth of *B. larvae* in the presence of yeast extract. No major difference between the response of the Ames and Sturtevant strains to the various salts was found.

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[1]Difco Laboratories, Inc., Detroit 1, Michigan.

Table 5. Effect of various salts on growth of *B. larvae*

Salts	Molar solution	Days of incubation					
		1		2		3	
		S[a]	Ames	S	Ames	S	Ames
KCl	0.10	3[b]	6	10	10	10	10
"	0.05	7	10	10	10	10	10
"	0.01	10	10	10	10	10	10
NaCl	0.10	5	9	10	10	10	10
"	0.05	6	10	10	10	10	10
"	0.01	9	8	10	10	10	10
Na_2HPO_4	0.10	0	0	0	3	7	10
"	0.05	0	0	2	10	10	10
"	0.01	9	6	9	10	10	10
KH_2PO_4	0.10	0	0	0	1	6	8
"	0.05	0	0	6	5	10	10
"	0.01	0	6	10	10	10	10
None	--	6	10	9	10	10	10

[a]Sturtevant strain.

[b]Number of positive tubes out of ten.

The medium used by Bailey and Lee (1962) contained one per cent glucose. This additional carbohydrate source did not seem necessary from results obtained with medium B in previous experiments. Nevertheless, various levels of

glucose were tested (Table 6) in medium B with and without added agar (0.2 per cent) to determine if the growth of B. larvae would be enhanced. Ten tubes of each medium were inoculated with about 10,000 cells of a 48 hour culture of B. larvae, either the Ames strain or Sturtevant strain. Only the results of the Ames strain are reported in Table 6 because the results with the Sturtevant strain were similar.

Table 6. Effect of glucose and agar on the growth of B. larvae

Per cent glucose	Per cent agar	Days of incubation		
		1	2	3
2.0	---	0[a]	0	8
2.0	0.2	0	0	0
1.0	---	10	10	10
1.0	0.2	0	1	10
0.5	---	10	10	10
0.5	0.2	0	9	10
0.0	---	10	10	10
0.0	0.2	10	10	10

[a]Number of tubes showing growth out of ten.

In the absence of added glucose, growth resulted in one day whether or not agar was included in the medium (Table

6). The presence of agar seemed to retard growth when 0.5 or 1.0 per cent glucose was used: ten tubes showed growth in one day when no agar was present; however, no tubes showed growth in one day when 0.2 per cent agar was included in the same media. This effect of agar was temporary since after three days of incubation most of the tubes were positive, except when the glucose level was 2.0 per cent. It is apparent that growth of *B. larvae* in medium B occurs even without glucose. Therefore, the use of 1.0 per cent glucose, as recommended by Bailey and Lee (1962) does not seem advisable in media for the cultivation of *B. larvae*. Furthermore, Katznelson and Lochhead (1944) reported that *B. larvae* survived longer when glucose was omitted from their peptone-yeast extract medium.

Previous results indicated that *B. larvae* could be grown in a number of media. Since many different protein sources have been used in media developed by various investigators, it was necessary to determine which source of organic nitrogen might be preferred for growth of *B. larvae*. Various media, listed in Table 6, were prepared by adding the appropriate quantities of nitrogen source to the following basal media:

Basal medium 1

Soluble starch 1.0 per cent
Glucose 0.5 per cent
KH_2PO_4, 0.01M, pH 6.6

Basal medium 2

Soluble starch	1.0 per cent
Glucose	0.5 per cent
Distilled water	

Each medium was dispensed in about four milliliter quantities in 13 x 120 millimeter tubes, sterilized, and inoculated with 1,000 cells of 48 hour culture of *B. larvae* grown in medium B without any agar. Although both the Ames and Sturtevant strains of *B. larvae* were tested for growth in all media, only the results of the Ames strain are shown since no difference could be found between the two strains. Two levels of nitrogen source, 1.0 and 0.5 per cent, were tested but only the results of 1.0 per cent level are shown because 0.5 per cent was too low a concentration.

Growth of *B. larvae* was absent or slight with added Brain Liver Heart, N-Z-Case, Bacto Peptone, Proteose Peptone, Brain Heart Infusion, Nutrient broth, Phytone, Trypticase and Beef Extract (Table 7). Therefore, these nitrogen sources were eliminated from further consideration. Bacto Casitone and Proteose Peptone showed promise, but these products were not investigated further because they are very similar to two other effective nitrogen sources, Proteose Peptone No. 3 and Proteose Peptone "C". A substantial quantity of growth was obtained when 1.0 per cent Difco Yeast Extract was incorporated into either basal medium. Since equivalent growth

Table 7. Effect of various nitrogen sources on growth of *B. larvae* in two different basal media

Nitrogen source	Basal medium	Days of incubation 1	2	3
Bacto Casitone[a]	1	10[b]	10	10
" "	2	10	10	10
Brain Liver Heart[a]	1	0	0	0
" " "	2	0	0	0
N-Z-Case[c]	1	0	0	0
" " "	2	0	0	0
Bacto Peptone[a]	1	0	3	4
" "	2	0	0	2
Proteose Peptone No. 3[a]	1	9	10	10
" "	2	10	10	10
Proteose Peptone[d]	1	9	10	10
" "	2	10	10	10
Proteose-Peptone "C"[d]	1	10	10	10
" " "	2	9	10	10
Trypticase[e]	1	0	0	0
"	2	3	10	10
Phytone[e]	1	0	0	0
"	2	0	0	0
Brain Heart Infusion[a]	1	0	0	3
" " "	2	0	1	2
Yeast Extract[a]	1	10	10	10
" "	2	10	10	10
Yeast Extract[e]	1	7	10	10
" "	2	0	5	9
Nutrient Broth[a]	1	0	0	2
" "	2	0	8	10
Beef Extract[a]	1	0	0	0
" "	2	0	2	4

[a]Difco Laboratories, Inc., Detroit 1, Michigan.

[b]Number of tubes showing growth out of ten.

[c]Sheffield Chemical, Norwich, New York.

[d]Case Laboratories, Chicago, Illinois.

[e]Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland.

was not obtained on another brand of yeast extract (Table 7), there was an indication that important differences in yeast extract from different sources existed.

A more definitive study was made using nitrogen sources selected on the basis of the results of the previous experiment. The composition of the four basal media used was as follows:

Basal medium 1

Soluble starch 1.0 per cent
Glucose 1.0 per cent
KH_2PO_4, 0.01M, pH 6.6

Basal medium 2

Soluble starch 1.0 per cent
Glucose 1.0 per cent
Distilled water

Basal medium 3

Soluble starch 1.0 per cent
KH_2PO_4, 0.01M, pH 6.6

Basal medium 4

Soluble starch 1.0 per cent
Distilled water

Procedures followed were identical to those given for the previous experiment, except that inocula of 1,000, 100, and 10 cells were used per tube. The inoculum was reduced in size because a smaller inoculum should place a more severe test on the efficacy of the medium. Only results of the inoculum size of 100 cells are shown because this inoculum gave the best differentiation.

Table 8. Effect of selected nitrogen sources on growth of *B. larvae* in four different basal media

Nitrogen source	Basal medium	Days of incubation			
		1	2	3	4
Proteose Peptone No. 3[a]	1	0[b]	0	0	5
" " " "	2	0	5	5	5
" " " "	3	0	0	5	5
" " " "	4	0	5	5	5
Proteose Peptone "C"[c]	1	0	0	0	0
" " "	2	0	0	0	0
" " "	3	0	0	0	0
" " "	4	0	0	0	0
Yeast extract[a]	1	0	0	5	5
" "	2	0	5	5	5
" "	3	0	5	5	5
" "	4	0	5	5	5
Yeast extract[d]	1	0	0	1	1
" "	2	0	0	0	0
" "	3	0	5	5	5
" "	4	0	5	5	5

[a]Difco Laboratories, Inc., Detroit 1, Michigan.

[b]Number of tubes showing growth out of five.

[c]Case Laboratories, Chicago, Illinois.

[d]Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland.

No growth occurred in Proteose Peptone "C" when the inoculum was reduced to 100 cells (Table 8); therefore, this peptone was eliminated from further consideration. Proteose Peptone No. 3 in basal media 2 and 4 supported good growth of *B. larvae*; however, Proteose Peptone No. 3 in basal medium

4 was selected because no glucose is used. An earlier experiment (Table 6) indicated that toxic products may possibly form when glucose and agar are autoclaved together in the same medium. This factor(s), if present, may interfere with the growth of _B. larvae_; therefore, basal medium 2 was not chosen.

Of the two sources of yeast extract tested, Difco Yeast Extract was superior in that all four basal media could be used with this product. Since difficulty was experienced in preliminary attempts to obtain cultures of _B. larvae_ on medium BA, it was felt that perhaps variation due to batch of yeast extract was responsible. However, three batches of Difco Yeast Extract were tested and were found to be equally satisfactory. No attempt was made to determine the reason for the brand-to-brand difference in yeast extract. Difco Yeast Extract with basal medium 1 was selected merely for comparative purposes because this is the medium used by Bailey and Lee (1962). Elimination of the glucose, which may be toxic when autoclaved with agar, results in medium BA. Basal medium 4 with Difco Yeast Extract is one of the media used by Foster _et al_. (1950) for demonstrating the role of starch in increasing the per cent sporulation of _B. larvae_.

The media that were apparently successful in supporting rapid growth of _B. larvae_ in broth were examined for their efficiency as plating media. Results shown in Table 6

indicated that the addition of agar might decrease the usefulness of the media for growth of *B. larvae*. The composition of the media used for plating was as follows:

Medium CA (Bailey and Lee, 1962)

Yeast extract[1]	1.0 per cent
Soluble starch	1.0 " "
Glucose	1.0 " "
Agar	1.5 " "
KH_2PO_4, 0.01M, pH 6.6	

Medium BA

Yeast extract[1]	1.0 per cent
Soluble starch	1.0 " "
Agar	1.5 " "
KH_2PO_4, 0.01M, pH 6.6	

Medium EA (Foster et al., 1950)

Yeast extract[1]	1.0 per cent
Soluble starch	1.0 " "
Agar	1.5 " "

Medium FA

Proteose Peptone No. 3[1]	1.0 per cent
Soluble starch	1.0 " "
Agar	1.5

Broths were prepared by omitting agar. Twenty-five milliliters of each broth medium were dispensed into each of four 125 milliliter Erlenmeyer flasks. Sturtevant and Ames strains of *Bacillus larvae* were subcultured twice in each medium. Each time, the culture was incubated for 48 hours.

[1]Difco Laboratories, Inc., Detroit 1, Michigan.

On the final subculture, each broth medium was plated into each of the four agar media. Plates were counted after incubation for 48 hours.

Table 9. Viable counts[a] of _B. larvae_ per milliliter of culture when grown on four different broths and plated into four agar media

Growth medium	Plating medium			
	CA	BA	EA	FA
C	10^4	2.3×10^7	1.9×10^7	3.8×10^6
B	7.8×10^5	1.3×10^7	7.2×10^6	5.9×10^5
E	2.3×10^5	7.1×10^6	5.5×10^6	4.8×10^6
F	1.1×10^6	2.0×10^7	1.5×10^7	4.2×10^6

[a]Counts are averages of two plates.

Resultant colonies on all of the media were very small; therefore, the plates were flooded with about two milliliters of a 0.1 per cent aqueous solution of 2, 3, 5-triphenyltetrazolium chloride, the plates were rocked gently, the excess fluid was poured off, and the plates were incubated at room temperature for about three hours (Solberg and Proctor, 1960). This enabled differentiation between the pinpoint bacterial colonies (red) from small particulate material in the media.

Medium CA was found to be a poor plating medium, but

medium C (no agar) was a good medium in which to propagate relatively large numbers of cells (Table 9). Medium FA was slightly better than medium CA as a plating medium for *B. larvae*, and like medium C, medium F was good for propagation. For all practical purposes, no difference was found between media B(BA) and E(EA).

It is apparent that further studies are needed if larger cell populations are to be attained *in vitro*. The highest count obtained was 2.3 x 10^7 vegetative cells per milliliter (Table 9). This is not necessarily the maximum population since counts were made after 48 hours and *B. larvae* is a slow grower as seen in the experiment on the Most Probable Numbers (Table 2). Mayer[1] found that *Bacillus mycoides* attained counts up to 10^8 cells per milliliter. Thus, it would seem possible that under proper growth conditions populations of 10^8 cells per milliliter should also be attained for *B. larvae*.

Viable counts of the Ames spore powder were about ten times higher that that of the older Sturtevant spore powder for an equivalent number of spores by direct microscopic counts. However, contamination of the spore powders, especially that of Sturtevant, made it impossible to obtain accurate counts. On the other hand, it is now possible to

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[1] Mayer, G. D., Iowa State University, Ames, Iowa. Maximum population of *Bacillus mycoides*. Private communication. 1963.

perform viable counts of *B. larvae* on relatively simple culture media. Such techniques can greatly facilitate the standardization of spore doses as reported in the latter part of this thesis or in studies of dosage-mortality such as those conducted by Hoage (1963).

In vitro spores used for *in vivo* studies (Figure 5) were prepared on medium BA. The type of culture vessel closure was found to be important for sporulation. *Bacillus larvae* sporulated better when airtight closures were used instead of cotton plugs. Sporulation could not be demonstrated in broth, but occurred in medium B containing 0.2 per cent agar. Attempts were unsuccessful to induce sporulation in medium B by the substitution of ascorbic acid, cysteine, or thioglycollic acid for agar. Sporulation of *B. larvae* was never found to occur in less than 48 hours in any of the media tested. Foster *et al.* (1950) found that sporulation of *B. larvae* occurred in four to five days with liver incorporated into the medium; however, without liver sporulation did not occur until about eight to 15 days after inoculation. No mention was made by Bailey and Lee (1962) of the time it took for *B. larvae* to commence sporulation. Tarr (1937a) routinely incubated cultures of *B. larvae* one week to obtain spores. Sporulation of recent *in vivo* isolates was found to show a high degree of sporulation (Tarr, 1937a). Tarr also reported that cultures maintained on

artificial medium lose the ability to form spores. In this author's experience, strains maintained on artificial medium for months sporulated equally as well as recent isolates.

There is much to be learned about the requirements for growth and sporulation of *B. larvae*. Strain differences may exist, thus results of various investigators also would differ.

An examination of *in vitro* and *in vivo* cells of *B. larvae* was made by electron microscopy for the first time, to the best of this author's knowledge, to determine if *B. larvae*, like *B. thuringiensis* (Figure 7), was crystalliferous. The electron micrographs were made through the courtesy of Roth[1]. No crystals were associated with spores of *B. larvae* from either *in vitro* or *in vivo* preparations.

In Vivo Studies of *Bacillus larvae*

Combs that showed a distilled water nonsurvival of greater than 10.99 per cent were excluded from statistical analyses. This procedure was followed because it was felt that high water losses indicated lack of complete control over all experimental conditions. Excess removal of control larvae on the part of the nurse bees might have been due to

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[1]Roth, L. E., Iowa State University, Ames, Iowa. 1961.

Figure 5. Electron micrograph of spores and vegetative cells of *B. larvae* grown on medium BA (3500X)

Figure 6. Electron micrograph of spores of *B. larvae* obtained from larvae dead of AFB (2900X)

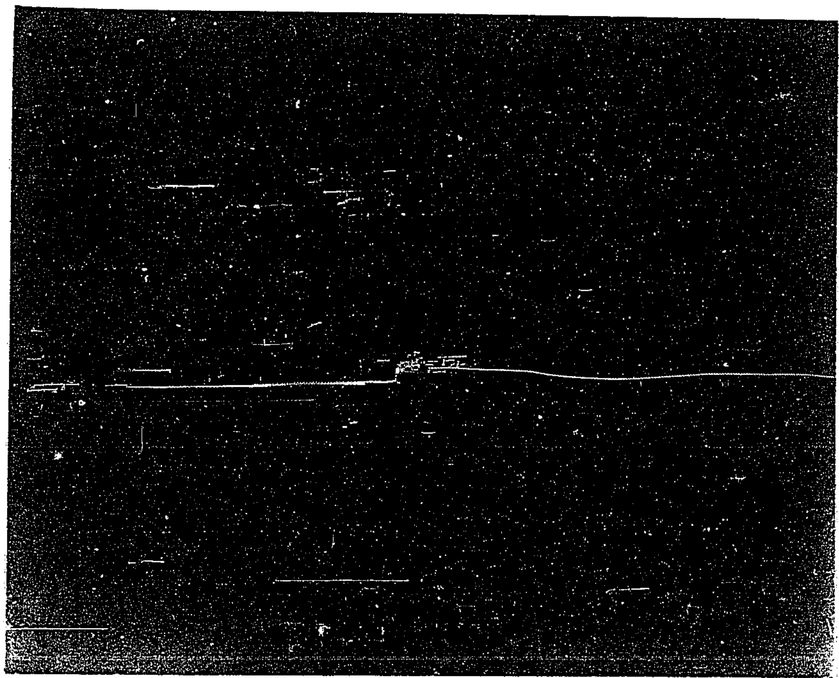
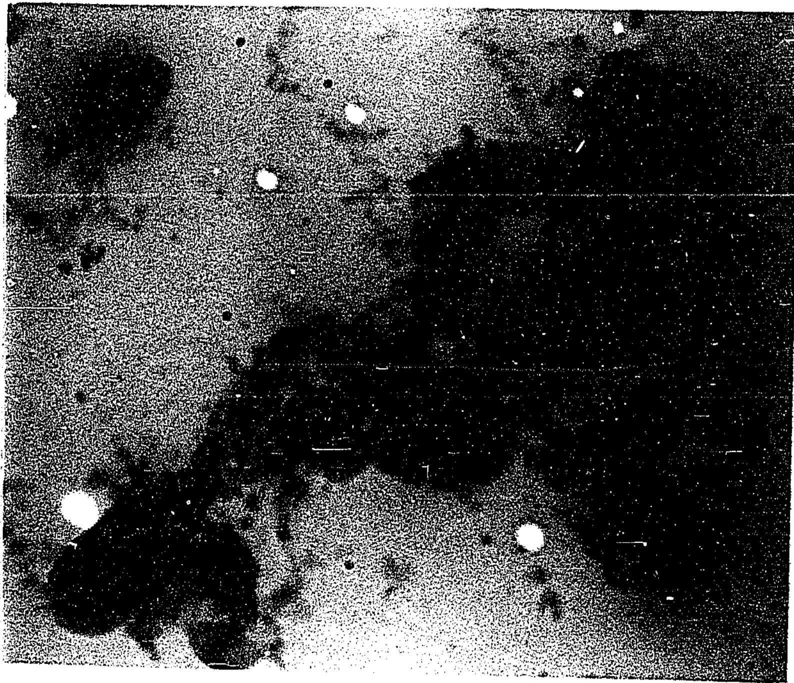


Figure 7. Electron micrograph of spores and crystals in a spore suspension of *B. thuringiensis* produced on Trypticase Soy Agar (3600X)



shortage of space for provisioning of honey and pollen or to the relatively small numbers of brood in the test combs. Another contributory factor, realized subsequent to these studies, is the possible effect contaminating materials in the distilled water might have on larval removal rates.

Per cent nonsurvival data were transformed by $\sqrt{X+1}$ for analyses. Pooled data were tested by analysis of variance using proportional sub-class numbers (Snedecor, 1956). When the data were amenable, the variance introduced by different queens and the interaction between queens and treatments were calculated. An example of the method of calculation is shown in the Appendix (Table 31).

In vitro spore suspensions are likely to be more homogeneous than spore preparations made from scales because a single colony (theoretically originating from one cell) can be isolated and a spore suspension prepared from it. To determine whether virulence of *in vitro* spores could be maintained, a spore suspension of *Bacillus larvae*, isolate "X", and the Ames spore powder were fed to 16 hour Brown resistant line larvae. As shown in Tables 10a and 10b, there was a highly significant difference in treatments. This difference was largely due to differences in nonsurvival between larvae fed spores and those fed water. The difference between the *in vitro* spores and the *in vivo* spores was found to be nonsignificant. Since isolate "X" had been

Table 10a. Comparative virulence of *B. larvae in vitro* isolate "X" versus Ames *in vivo* spores in 16 hour Brown resistant line. Pooled data[a]

Treatment	Spore dosage	Base count	Nonsurvivors	Per cent nonsurvival
Water	--	59	1	1.69
"X"	15,000	59	55	93.22
Ames	15,000	61	57	93.44

[a]See Appendix, Table 32 for raw data.

Table 10b. Analysis of variance of *in vitro* "X" spores in 16 hour resistant line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	87.76	43.88	137.12**
Water versus spores	(1)	(87.75)	87.75	274.22**
"X" versus Ames	(1)	(0.01)	0.01	0.03
Error	3	0.97	0.32	

**Indicates significance at the 0.01 level of probability.

maintained on an artificial medium for over five months prior to preparation of the *in vitro* spore suspension, the virulence of *B. larvae* apparently is not affected by cultivation exterior to the host on the laboratory medium used in this work. This finding is in agreement with the observations of Ark (1937) who reported that virulent cultures of *Erwinia amylovora* were still virulent even after being cultivated repeatedly on a laboratory medium for four years. Maintenance of the virulence in the present study seems to indicate that medium BA is satisfactory for preparation of *B. larvae* spore suspensions; however, five months may not be a sufficiently long period to determine whether a culture would ultimately lose its virulence if cultivated *in vitro* for an extended period.

Two single colonies were selected at random by plating the Ames spore powder on medium BA. Since 15,000 spores per larva of the Ames spore powder was an overwhelming dose (Table 10a) it was hoped that, by use of a smaller inoculum, differences could be noted that otherwise might remain undetected. A highly significant difference was found between the two random isolates from the Ames spore powder (Table 11b). Isolate 1c was found to be more virulent than isolate 2c (Table 11a). This difference in virulence among isolates from a common stock is not unusual. Virulence of a particular culture is a function of the proportion of

Table 11a. Comparative virulence of two *in vitro* isolates of the Ames spore powder. Pooled data

Treatment	Spore dosage	Base count	Nonsurvivors	Per cent nonsurvival
Water	---	233	11	4.72
1c	500	261	170	65.13
2c	500	250	64	25.60

Table 11b. Analysis of variance of Ames *in vitro* isolates 1c versus 2c in 24 hour susceptible line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	150.63	75.32	60.74**
Water versus spores	(1)	(111.75)	111.75	90.12**
1c versus 2c	(1)	(38.88)	38.88	31.35**
Queens	4	5.23	1.31	1.06
Queens x treatments	8	7.33	0.92	0.74
Error	9	11.14	1.24	

virulent to avirulent cells (Lincoln, 1940). As would be expected, no significant difference was found among the queens (Table 11b). The interaction between queens and treatments was likewise found to be nonsignificant.

Two randomly selected colonies from the Sturtevant spore powder were used to prepare spore suspensions 1a and 2b. These were then fed to 24 hour Van Scoy line larvae. An apparent difference in nonsurvival between spore suspensions 1a and 2b was not statistically significant (Tables 12a and 12b). It is interesting to note that, of the four *in vitro* spore suspensions, Ames 1c was the highest and Ames 2c was the lowest in virulence. The two Sturtevant *in vitro* spore suspensions fell between the two Ames *in vitro* spore preparations in virulence (Tables 11a and 12a).

A spore suspension (SF) was obtained from remains of F_1 larvae (Brown x Van Scoy) which were fed 15,000 Sturtevant spores. Sixteen hour Brown larvae and 24 hour Van Scoy larvae were used for this second passage. To observe whether the virulence of these SF spores had changed, Sturtevant spores were also fed. The dosage was standardized at 15,000 spores per larva. The second serial passage affected an apparent reduction in virulence (Table 13); however, insufficient data were obtained for statistical analysis. Spores from the Brown line were designated SFB and those from the Van Scoy line SFV.

Table 12a. Comparative virulence of two *in vitro* isolates of the Sturtevant spore powder. Pooled data

Treatment	Spore dosage	Base count	Nonsurvivors	Per cent nonsurvival
Water	---	268	4	1.49
1a	500	312	127	40.70
2b	500	302	165	54.64

Table 12b. Analysis of variance of Sturtevant *in vitro* isolates 1a versus 2b in 24 hour susceptible line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	200.98	100.49	104.68**
Water versus spores	(1)	(197.40)	197.40	205.62**
1a versus 2b	(1)	(3.58)	3.58	3.73
Queens	4	6.60	1.65	1.72
Queens x treatments	8	8.54	1.07	1.11
Error	15	14.46	0.96	

Table 13. Serial passage number two of spore suspension SF

Line	Age	Treatment	Base count	Nonsurvivors	Per cent nonsurvival
Brown	16	Water	54	3	5.56
		SF	48	21	43.75
		Sturtevant	48	37	47.08
Van Scoy	24	Water	48	4	8.33
		SF	40	17	42.50
		Sturtevant	44	29	65.91

Both SFB and SFV spore suspensions were fed to 16 and 24 hour Brown and Van Scoy line larvae at the level of 15,000 spores per larva. There were no statistically significant differences in the virulence of the spores obtained from the susceptible or resistant lines of bees. Spores obtained from the Van Scoy larvae inoculated at 24 hours of age were just as virulent for the Van Scoy line as spores from the 16 hour Brown line (Tables 14a, 14b, 15a, 15b, and 15c). Data from the 24 hour Van Scoy line were not statistically analyzed due to the lack of sufficient data. Subsequently, spores from AFB dead 24 hour Van Scoy line (SFVV) and AFB dead 16 hour Brown line (SFBB) were fed to 16 and 24 hour Brown line larvae.

In both the 16 and 24 hour Brown line larvae no

Table 14a. Serial passage number three in Van Scoy line larvae. Pooled data

Inoculation age (hours)	Treatment	Base count	Nonsurvivors	Per cent nonsurvival
16	Water	90	5	5.56
	SFV	103	98	95.14
	SFB	107	100	93.46
24	Water	42	0	0.00
	SFV	36	31	86.11
	SFB	44	42	95.45

Table 14b. Analysis of variance of SFV versus SFB in 16 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F
Treatments	2	102.30	51.15	1023.00**
Water versus spores	(1)	(102.29)	102.29	2045.80**
SFV versus SFB	(1)	(0.01)	0.01	0.20
Queens	1	0.30	0.30	6.00
Queens x treatments	2	0.52	0.26	5.20
Error	3	0.14	0.05	

Table 15a. Serial passage number three in Brown line larvae. Pooled data

Age in hours	Treatments	Base count	Nonsurvivors	Per cent nonsurvival
16	Water	141	10	7.09
	SFV	181	173	95.58
	SFB	152	146	96.05
24	Water	95	7	7.37
	SFV	88	79	89.77
	SFB	112	91	81.25

Table 15b. Analysis of variance of SFV versus SFB in 16 hour Brown line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	133.63	66.82	514.00**
Water versus spores	(1)	(133.62)	133.62	1027.85**
SFV versus SFB	(1)	(0.01)	0.01	0.07
Queens	1	0.17	0.17	1.31
Queens x Treatments	2	0.07	0.04	0.31
Error	6	0.80	0.13	

Table 15c. Analysis of variance of SFV versus SFB in 24 hour Brown line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	58.43	29.22	100.76**
Water versus spores	(1)	(58.39)	58.39	201.34**
SFV versus SFB	(1)	(0.04)	0.04	0.14
Error	3	0.87	0.29	

Table 16a. Serial passage number three in Brown line larvae. Five-hundred spores per larva. Pooled data

Age in hours	Treatment	Base count	Nonsurvivors	Per cent nonsurvival
16	Water	148	10	6.76
	SFVV	149	141	94.63
	SFBB	155	144	92.90
24	Water	140	10	7.14
	SFVV	122	93	76.23
	SFBB	149	116	77.85

Table 16b. Analysis of variance of SFVV versus SFBB in 16 hour Brown line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	64.55	32.28	215.20**
Water versus spores	(1)	(64.54)	64.54	430.27**
SFVV versus SFBB	(1)	(0.01)	0.01	0.07
Error	3	0.45	0.15	

Table 16c. Analysis of variance of SFVV versus SFBB in 24 hour Brown line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	66.92	33.46	49.20**
Water versus spores	(1)	(66.70)	66.70	98.09**
SFVV versus SFBB	(1)	(0.22)	0.22	0.32
Error	6	4.08	0.68	

significant difference could be shown between SFVV and SFBB (Tables 16a, 16b, and 16c). Virulence of B. larvae was not altered by genetic constitution of the host, whether resistant or susceptible. It should be noted, however, that the ages of larvae through which the pathogen was passed differed in the two (resistant and susceptible) lines of bees. Larval ages at time of inoculation were selected to obtain approximately the same degree of kill in both lines of bees, and the two lines might well possess the same degree of resistance of susceptibility at the ages tested. To determine possible changes in virulence of SFVV from the original Sturtevant spore powder, both suspensions were fed to 16 and 24 hour Van Scoy and Brown line larvae. The dosage employed was 15,000 spores per larva.

A difference of about 14 per cent nonsurvival was found in the 16 hour Van Scoy larvae between Sturtevant and SFVV spores (Table 17a). In the 24 hour Van Scoy larvae a difference of 32 per cent was noted. This difference is almost twice as large as that observed when 16 hour Van Scoy larvae received the same spore preparation. The difference in per cent nonsurvival between 16 and 24 hour Van Scoy larvae fed Sturtevant spores was 23 per cent, whereas the difference in nonsurvival in larvae fed SFVV was only five per cent. Differences in resistance of the host were not as marked when Sturtevant spores were used because the

Table 17a. Comparative virulence of SFVV and Sturtevant spore powder in Van Scoy line larvae. Pooled data

Age in hours	Treatment	Base count	Nonsurvivors	Per cent nonsurvival
16	Water	211	11	5.21
	Sturtevant	217	178	82.03
	SFVV	214	205	95.79
24	Water	109	8	7.34
	Sturtevant	111	65	58.56
	SFVV	126	114	90.48

inoculum of 15,000 spores of SFVV was apparently an overwhelming dose (Table 17a). Very high nonsurvival rates were experienced with SFVV in both ages of Van Scoy larvae tested.

Differences in per cent nonsurvival between larvae fed Sturtevant or SFVV spores were found to be highly significant in both 16 and 24 hour Van Scoy line larvae (Tables 17b and 17c). A significant difference in queens was also evident in both the 16 and 24 hour larvae. This could be due to differences in the time when each queen initiates egg laying. When a queen is nervous, she may delay egg laying until late in the six hour caging period. On the other hand, another queen might commence egg laying immediately after being caged. Therefore, this difference

Table 17b. Analysis of variance of Sturtevant spore powder versus SFVV in 16 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	234.57	117.28	533.09**
Water versus spores	(1)	(232.65)	232.65	1057.50**
Sturtevant versus SFVV	(1)	(1.92)	1.92	8.73**
Queens	1	1.33	1.33	6.04*
Queens x treatments	2	0.38	0.19	0.86
Error	15	3.28	0.22	

*Indicates significance at the 0.05 level of probability.

Table 17c. Analysis of variance of Sturtevant spore powder versus SFVV in 24 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	120.91	60.46	755.75**
Water versus spores	(1)	(112.17)	112.17	1402.12**
Sturtevant versus SFVV	(1)	(8.74)	8.74	10.92**
Queens	1	0.76	0.76	9.50*
Queens x treatments	2	0.12	0.06	
Error	9	0.74	0.08	

among queens may be a reflection of the interaction between colony disposition and queen behavior.

A difference of about 21 per cent was found in nonsurvival rates between 16 hour Brown larvae fed Sturtevant or SFVV spores (Table 18a). A much larger difference, about 48 per cent, was found in the 24 hour Brown larvae (Table 18a). The difference in per cent nonsurvival between 16 and 24 hour Brown line larvae fed Sturtevant spores was nearly 26 per cent. In the 16 and 24 hour Brown larvae fed SFVV spores, the per cent nonsurvival in the 24 hour larvae was 1.62 per cent greater than that of the 16 hour larvae.

Table 18a. Comparative virulence of SFVV and Sturtevant spore powder in Brown line larvae. Pooled data

Age in hours	Treatment	Base count	Nonsurvivors	Per cent nonsurvival
16	Water	146	10	6.85
	Sturtevant	131	94	71.76
	SFVV	134	124	92.54
24	Water	136	9	6.62
	Sturtevant	139	64	46.04
	SFVV	137	129	94.16

Table 18b. Analysis of variance of Sturtevant spore powder versus SFVV in 16 hour Brown line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F
Treatments	2	83.57	41.78	696.33**
Water versus spores	(1)	(81.67)	81.67	1361.17**
Sturtevant versus SFVV	(1)	(1.90)	1.90	31.67*
Queens	1	0.06	0.06	1.00
Queens x treatments	2	0.08	0.04	0.67
Error	3	0.19	0.06	

Table 18c. Analysis of variance of Sturtevant spore powder versus SFVV in 24 hour Brown line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatment	2	73.24	36.62	366.20**
Water versus spores	(1)	(60.83)	60.83	608.30**
Sturtevant versus SFVV	(1)	(12.41)	12.41	124.10**
Error	6	0.58	0.10	

The difference between Sturtevant and SFVV spores was found to be significant at 0.05 level in the 16 hour Brown line larvae. The difference in the 24 hour Brown line larvae was significant at the 0.01 level (Tables 18b and 18c). Therefore, the virulence of the serially passed SFVV spores was found to be higher in all cases than the parent Sturtevant spore powder.

Differences in per cent nonsurvival could be seen between lines and ages only when Sturtevant spores were used. The per cent nonsurvival due to feeding 15,000 Sturtevant spores in 16 hour Van Scoy larvae was 82.03 per cent, in 16 hour Brown larvae 71.76 per cent, in 24 hour Van Scoy larvae 58.56 per cent and in 24 hour Brown larvae 46.04 per cent. The nonsurvival due to feeding 15,000 SFVV spores ranged from 90.48 - 95.79 per cent between all lines and ages, whereas nonsurvival due to feeding 15,000 Sturtevant spores ranged from 46.04 - 82.03 per cent (Tables 17a and 18a).

The virulence of SFBB spores was found to be similar to that of the SFVV preparation (Table 16a). To measure the change in virulence of SFBB spores both the parent Sturtevant spore powder and SFBB spores were compared at the level of 15,000 spores per larva. Sixteen and 24 hour larvae of Van Scoy and Brown lines were used for the comparisons. The virulence of the Sturtevant spore powder was consistently lower than that of SFBB (Table 19a). Per cent nonsurvival

Table 19a. Comparative virulence of SFBB and Sturtevant spore powder in Van Scoy line larvae. Pooled data

Age in hours	Treatments	Base count	Nonsurvivors	Per cent nonsurvival
16	Water	148	10	6.76
	Sturtevant	137	105	76.64
	SFBB	126	122	96.82
24	Water	124	7	5.64
	Sturtevant	124	86	69.35
	SFBB	144	139	96.53

Table 19b. Analysis of variance of Sturtevant spore powder versus SFBB in 16 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	118.80	59.40	371.25**
Water versus spores	(1)	(116.73)	116.73	729.56**
Sturtevant versus SFBB	(1)	(2.07)	2.07	12.94*
Queens	1	0.02	0.02	0.12
Queens x treatments	2	0.83	0.42	2.62
Error	6	0.94	0.16	

Table 19c. Analysis of variance of Sturtevant spore powder versus SFBB in 24 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	127.05	63.52	124.55**
Water versus spores	(1)	(122.45)	122.45	240.10**
Sturtevant versus SFBB	(1)	(4.60)	4.60	9.02*
Queens	1	0.33	0.33	0.65
Queens x treatments	2	0.20	0.10	0.20
Error	6	3.06	0.51	

due to Sturtevant spore powder was about 20 per cent less in the 16 hour Van Scoy larvae and some 27 per cent less in the 24 hour Van Scoy larvae than with the SFBB preparations. There was a seven per cent difference in the per cent nonsurvival between 16 and 24 hour Van Scoy larvae that were fed Sturtevant spores. The difference in per cent nonsurvival in 16 and 24 hour Van Scoy larvae fed SFBB was less than one per cent (Table 19a).

A statistically significant difference was found between Sturtevant spores and the serially passed spores SFBB. This difference was significant at the 0.05 level in both 16 and 24 hour Van Scoy larvae. Neither the variation due to queens nor the interaction between queens and treat-

ments was significant.

The serially passed spores, SFBB, were found to be more virulent than the parent Sturtevant spores. The difference in per cent nonsurvival in 16 hour Brown line larvae fed Sturtevant and SFBB spores was about 31 per cent. In the 24 hour Brown line larvae, the difference was about 47 per cent (Table 20a). A 24 per cent reduction in per cent nonsurvival was seen between 16 and 24 hour Brown line larvae when Sturtevant spores were fed. This marked difference due to age was not evident when SFBB spores were fed.

Table 20a. Comparative virulence of SFBB and Sturtevant spore powder in Brown line larvae. Pooled data

Age in hours	Treatment	Base count	Non-survivors	Per cent nonsurvival
16	Water	137	8	5.84
	Sturtevant	140	92	65.71
	SFBB	137	132	96.35
24	Water	154	13	8.44
	Sturtevant	187	78	41.71
	SFBB	185	164	88.65

Statistically significant differences between the parent Sturtevant and SFBB spores were found in both 16 and

Table 20b. Analysis of variance of Sturtevant spore powder versus SFBB in 16 hour Brown line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	56.92	28.46	284.60**
Water versus spores	(1)	(54.32)	54.32	543.20**
Sturtevant versus SFBB	(1)	(2.60)	2.60	26.00*
Error	3	0.30	0.10	

Table 20c. Analysis of variance of Sturtevant spore powder versus SFBB in 24 hour Brown line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	63.22	31.61	121.58**
Water versus spores	(1)	(50.63)	50.63	233.19**
Sturtevant versus SFBB	(1)	(12.59)	12.59	48.42**
Error	7	1.86	0.26	

24 hour Brown line larvae (Tables 20b and 20c). The difference in the 16 hour Brown line larvae was significant at the 0.05 level, while in the 24 hour Brown line larvae the difference was significant at the 0.01 level.

On the basis of the data presented thus far, one must conclude that virulence of the Sturtevant spore powder was increased by three serial passages. The first passage was through a F_1 line which was intermediate in resistance. The second and third passages were through 24 hour susceptible line larvae. A similar increase in virulence was noted when Sturtevant spores, obtained from F_1 larvae dead of AFB, were serially passed twice in 16 hour resistant line larvae. The SFVV and SFBB spores were equally virulent. Therefore, selection for virulence was not line specific: in other words, serial passage through Brown line larvae did not result in the selection of a strain of increased virulence only to Brown larvae and not to Van Scoy line larvae. Likewise, serial passage through the Van Scoy line larvae did not select a strain more virulent only to Van Scoy line larvae.

The role of the immune bodies in resistant bees reported by Gary _et al_. (1948) could possibly be a factor in the selection of a virulent progeny. Zelle (1942) concluded that the virulence of _Salmonella typhimurium_ could be increased by passage through either susceptible or resistant

mice. He attributed this increase in pathogenicity to selection of strains of increased virulence. Gowan (1945) observed that resistant and susceptible chicks were selective for the more virulent cells of _Shigella gallinarum_. Thus, the less virulent cells in the population of _B. larvae_ spores could germinate and multiply but the host, irregardless of resistance or susceptibility, could select for the more virulent cells.

The number of viable Sturtevant spores, using direct microscopic counts as a basis, was only one-tenth of the more virulent Ames spore powder in medium EA. Therefore, serial passage could result in the production of a spore population with a higher per cent germination than the older Sturtevant preparation. The net effect of increased viability would be a higher dosage which in turn would be reflected by higher per cent nonsurvival.

Since no decrease in virulence could be demonstrated upon passage of _B. larvae_ through susceptible or resistant lines of honey bees at ages where susceptibility to the pathogen was at approximately the same level, it was of interest to discover if age of the host would influence the virulence of the pathogen. Therefore, six and 40 hour Van Scoy line larvae were both fed 15,000 Sturtevant spores for the first passage. There was a definite decrease in the per cent nonsurvival due to age. Table 21a shows that 87.83

per cent of 16 hour Van Scoy larvae did not survive as opposed to only 13.66 per cent of the 40 hour Van Scoy larvae. Statistically significant differences were found in treatments. The significance in the six hour Van Scoy larvae was at the level of 0.05 as opposed to the significance at 0.01 in the 40 hour Van Scoy larvae. The queens were significant at the 0.05 level in the 40 hour Van Scoy larvae. Interaction between queens and treatments was not significant in both cases (Tables 21b and 21c).

Spores from AFB dead larvae inoculated at six hours (SY-1) and spores from larvae inoculated at 40 hours (SO-1) were fed to six and 40 hour Van Scoy line larvae at the level of 15,000 spores per larva. As shown in Table 22a, spore suspension SY-1 resulted in a nonsurvival of 92.36 per cent of the six hour Van Scoy larvae. The SO-1 spore suspension in 40 hour Van Scoy larvae resulted in 7.62 per cent nonsurvival. Both the SY-1 and SO-1 spore suspensions were found to result in a highly significant nonsurvival percentage. The variation due to queens was significant at the 0.05 level in the six hour larvae but not in the 40 hour larvae. Interaction between queens and treatments was nonsignificant in both cases.

For the comparison of virulence, spores from larvae dead of AFB after feeding SY-1 were labelled SY-2L and spores from pupae dead of AFB were designated SY-2P. Spores

Table 21a. Serial passage number one. Comparative virulence of Sturtevant spore powder in Van Scoy line larvae. Pooled data

Age in hours	Treatment	Base count	Nonsurvivors	Per cent nonsurvival
6	Water	58	2	3.45
	Sturtevant	115	101	87.83
40	Water	168	5	2.98
	Sturtevant	366	50	13.66

Table 21b. Analysis of variance of Sturtevant spore powder in six hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	1	106.50	106.50	76.07*
Queens	2	0.77	0.39	0.28
Queens x treatments	2	1.46	0.73	0.52
Error	2	2.79	1.40	

Table 21c. Analysis of variance of Sturtevant spore powder in 40 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	1	11.63	11.63	18.46**
Queens	2	8.49	4.30	6.82*
Queens x treatments	2	0.90	0.45	0.71
Error	10	6.26	0.63	

Table 22a. Serial passage number two. Comparative virulence of Sturtevant spore powder in Van Scoy line larvae. Pooled data

Age in hours	Treatment	Base count	Nonsurvivors	Per cent nonsurvival
6	Water	130	7	5.38
	SY-1	288	266	92.36
40	Water	216	2	1.00
	SO-1	446	34	7.62

Table 22b. Analysis of variance of SY-1 spores in six hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	1	130.03	130.03	6501.50**
Queens	3	2.42	0.81	40.50*
Queens x treatments	3	1.08	0.36	18.00
Error	2	0.04	0.02	

Table 22c. Analysis of variance of SO-1 spores in 40 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	1	7.78	7.78	20.47**
Queens	3	4.91	1.64	0.62
Treatments x queens	3	4.74	1.58	0.60
Error	10	26.34	2.63	

from larvae fed SO-1 were designated SO-2L and spores from pupae were labelled SO-2P. All four spore suspensions were tested in 24 hour Van Scoy larvae at the level of 500 spores per larva. The results are given in Table 23a. A significant difference at the 0.05 level between spore suspension SY-2L and SY-2P was found (Table 23b). The variation of the queens was likewise significant at the 0.05 level. In the opinion of the investigator, the difference in nonsurvival due to SY-2L and SY-2P is in error. This could very well be due to experimental error. If age was an important factor, this same difference in virulence would have been apparent in the spores from the 40 hour larvae. Likewise, a greater difference would be expected between spores passed in six hour larvae and spores passed in 40 hour larvae.

Sturtevant spores serially passed in 40 hour Van Scoy larvae showed no difference in the virulence of the spores whether from larvae or pupae. The per cent nonsurvival was similar (Table 24a). A statistically significant difference was found between spores and water but no statistically significant difference between SO-2L and SO-2P. Interaction and queens were also found to be nonsignificant.

Since the virulence of Sturtevant spores can be increased by serial passage in both resistant and susceptible lines of larvae (Tables 17a and 18a), it was of interest to determine whether the virulence of Ames spores could also be

Table 23a. Comparative virulence of Sturtevant spores serially passed in six hour Van Scoy line larvae. Pooled data

Treatment	Spore dosage	Base count	Nonsurvivors	Per cent nonsurvival
Water	---	237	15	6.33
SY-2L	500	262	207	79.01
SY-2P	500	259	236	91.12

Table 23b. Analysis of variance of Sturtevant spores from larvae and pupae dead of AFB

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	207.24	103.62	471.00**
Water versus spores	(1)	(205.75)	205.75	935.23**
SY-2L versus SY-2P	(1)	(1.49)	1.49	6.77*
Queens	2	2.65	1.32	6.00*
Queens x treatments	4	0.39	0.10	0.45
Error	12	2.65	0.22	

Table 24a. Comparative virulence of Sturtevant spores serially passed in 40 hour Van Scoy line larvae. Pooled data

Treatment	Spore dosage	Base count	Nonsurvivors	Per cent nonsurvival
Water	---	523	30	5.74
SO-2L	500	539	459	85.16
SO-2P	500	566	492	86.92

Table 24b. Analysis of variance of Sturtevant spores from larvae and pupae dead of AFB

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	460.97	230.48	886.46**
Water versus spores	(1)	(460.90)	460.90	1772.69**
SO-2L versus SO-2P	(1)	(0.07)	0.07	0.27
Queens	4	0.46	0.12	0.46
Queens x treatments	8	1.57	0.20	0.77
Error	30	7.84	0.26	

increased. Therefore, spores of Sturtevant or Ames spore powder were fed to 24 hour Van Scoy line larvae.

A nonsurvival of 53.12 per cent was obtained from 24 hour Van Scoy line larvae fed Sturtevant spores; whereas the nonsurvival due to Ames spores was 92.66 per cent in the 24 hour Van Scoy larvae (Table 25a). This difference was significant at the 0.01 level (Table 25b). Queens and interaction were found to be nonsignificant. Spores from larvae dead after feeding Sturtevant spores were labelled SV and thos dead after feeding Ames spores AV. The change in virulence was determined by feeding Sturtevant spores and SV spores to 24 hour Van Scoy line larvae. Ames spores and SV spores were also compared in 24 hour Van Scoy line larvae. The virulence of the Sturtevant spore powder was increased by one passage in 24 hour Van Scoy line larvae (Table 26a). The difference between Sturtevant and SV in per cent nonsurvival was about 47 per cent. This difference was found to be significant at the 0.01 level (Table 26b). Variation due to queens was significant at the 0.01 level but the interaction between queens and treatments was not significant (Table 26b). An increase in virulence of about 30 per cent was observed when Ames spores were passed once in 24 hour Van Scoy line larvae (Table 27a). This increase in virulence of AV over the original Ames spores was highly significant (Table 27b). The variation of the queens and the

Table 25a. Comparative virulence of Sturtevant and Ames spore powder. Pooled data

Treatment	Spore dosage	Base count	Nonsurvivors	Per cent nonsurvival
Water	---	119	5	4.20
Sturtevant	15,000	128	68	53.12
Ames	15,000	109	101	92.66

Table 25b. Analysis of variance of Sturtevant versus Ames spore powder in 24 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatment	2	174.08	87.04	128.00**
Water versus spores	(1)	160.15	160.15	235.51**
Sturtevant versus Ames	(1)	13.93	13.93	20.48**
Queens	2	1.80	0.90	1.32
Queens x treatments	4	1.98	0.50	0.74
Error	9	6.08	0.68	

Table 26a. Virulence of Sturtevant and SV spores in 24 hour Van Scoy line larvae. Pooled data

Treatment	Spore dosage	Base count	Nonsurvivors	Per cent nonsurvival
Water	---	260	11	4.23
Sturtevant	500	287	55	19.16
SV	500	287	191	66.55

Table 26b. Analysis of variance of Sturtevant versus SV spores in 24 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	184.51	92.26	439.33**
Water versus spores	(1)	(112.42)	112.42	535.33**
Sturtevant versus SV	(1)	(72.09)	72.09	343.29**
Queens	4	7.58	1.90	9.05**
Queens x treatments	8	4.11	0.51	2.43
Error	15	3.18	0.21	

Table 27a. Virulence of Ames and AV spores in 24 hour Van Scoy line larvae. Pooled data

Treatment	Spore dosage	Base count	Nonsurvivors	Per cent nonsurvival
Water	---	482	30	6.22
Ames	500	514	299	58.17
AV	500	498	440	88.35

Table 27b. Analysis of variance of Ames versus AV spores in 24 hour Van Scoy line larvae

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	320.15	160.08	242.54**
Water versus spores	(1)	(296.48)	296.48	449.21**
Ames versus AV	(1)	(23.67)	23.67	35.86**
Queens	3	0.10	0.03	0.04
Queens x treatments	6	3.31	0.55	0.83
Error	27	17.86	0.66	

interaction were both nonsignificant.

The virulence of the Ames and Sturtevant spore powder was markedly increased by one serial passage in 24 hour Van Scoy line larvae. In fact, all serial passages, whether the host was resistant or susceptible, resulted in an ultimate increase in virulence.

In Vivo Studies of *Bacillus thuringiensis*

Although *B. thuringiensis* is not a known pathogen of the honey bee, it was of interest to determine the potential effects of this bacterium on honey bee populations. A comparison of the effect of *B. thuringiensis* and *B. larvae* (Ames spore powder) was made by feeding 1800 spores to lines of bees resistant and susceptible to AFB.

The data in Tables 28a and 28b were not statistically analyzed due to the high degree of variation in the few combs that had a control mortality of less than 10.99 per cent. *Bacillus thuringiensis* did not appear pathogenic to either AFB resistant or susceptible lines of bees of 16, 24, and 32 hours of age. Larvae remaining in the cells showed no symptoms of disease after being fed 1800 spores of *B. thuringiensis*, whereas larvae fed spores of *B. larvae* showed characteristic symptoms of AFB.

Lecomte and Martouret (1959) concluded that *B.*

Table 28a. Effect of feeding *B. thuringiensis* and *B. larvae* to Brown line larvae. Pooled data[a]

Age in hours	Treatments	Base count	Missing	Non-survivors	Per cent nonsurvival
16	Water	55	4	0	7.27
	BT[b]	69	7	0	10.14
	Ames	70	29	32	87.14
24	Water	88	4	0	4.54
	BT	80	6	0	7.50
	Ames	89	23	30	59.64
32	Water	132	1	1	1.51
	BT	128	7	0	5.46
	Ames	128	5	10	11.71

[a]Raw data in Appendix Table 33.

[b]*Bacillus thuringiensis*.

thuringiensis was only slightly toxic to adult honey bees. Wilson (1962) reported that *B. thuringiensis* appears to be completely safe to the honey bees. Whereas Lecomte and Martouret (1959) studied only the adult honey bees, Wilson (1962) studied the total effect of *B. thuringiensis* on honey bee colonies. Nevertheless, both studies led to the same conclusion.

In the course of conducting these experiments, a

Table 28b. Effect of feeding B. thuringiensis and B. larvae to Van Scoy line larvae. Pooled data

Age in hours	Treatments	Base count	Missing	Non-survivors	Per cent nonsurvival
16	Water	118	5	1	5.08
	BT	90	6	2[a]	8.88
	Ames	141	83	49	93.61
24	Water	79	5	1	7.59
	BT	65	3	2[a]	7.69
	Ames	74	39	23	83.78
32	Water	106	8	0	7.54
	BT	108	11	0	10.18
	Ames	96	30	26	58.33

[a]AFB dead, confirmed by smear of larva.

high rate of removal due to B. thuringiensis was observed. Thus, selected combs of 16, 24, and 32 hour Brown and Van Scoy larvae were observed at intervals of two days, after a one day count, to determine the removal rates of larvae. Figures 8, 9, 10, 11, 12 and 13 are results averaged from at least three separate combs and are based on over 100 larvae per treatment-age group. All combs, regardless of control mortality greater than 10.99 per cent, were used for these analyses. The raw data can be seen in the

Figure 8. Removal rate of 16 hour Brown line larvae

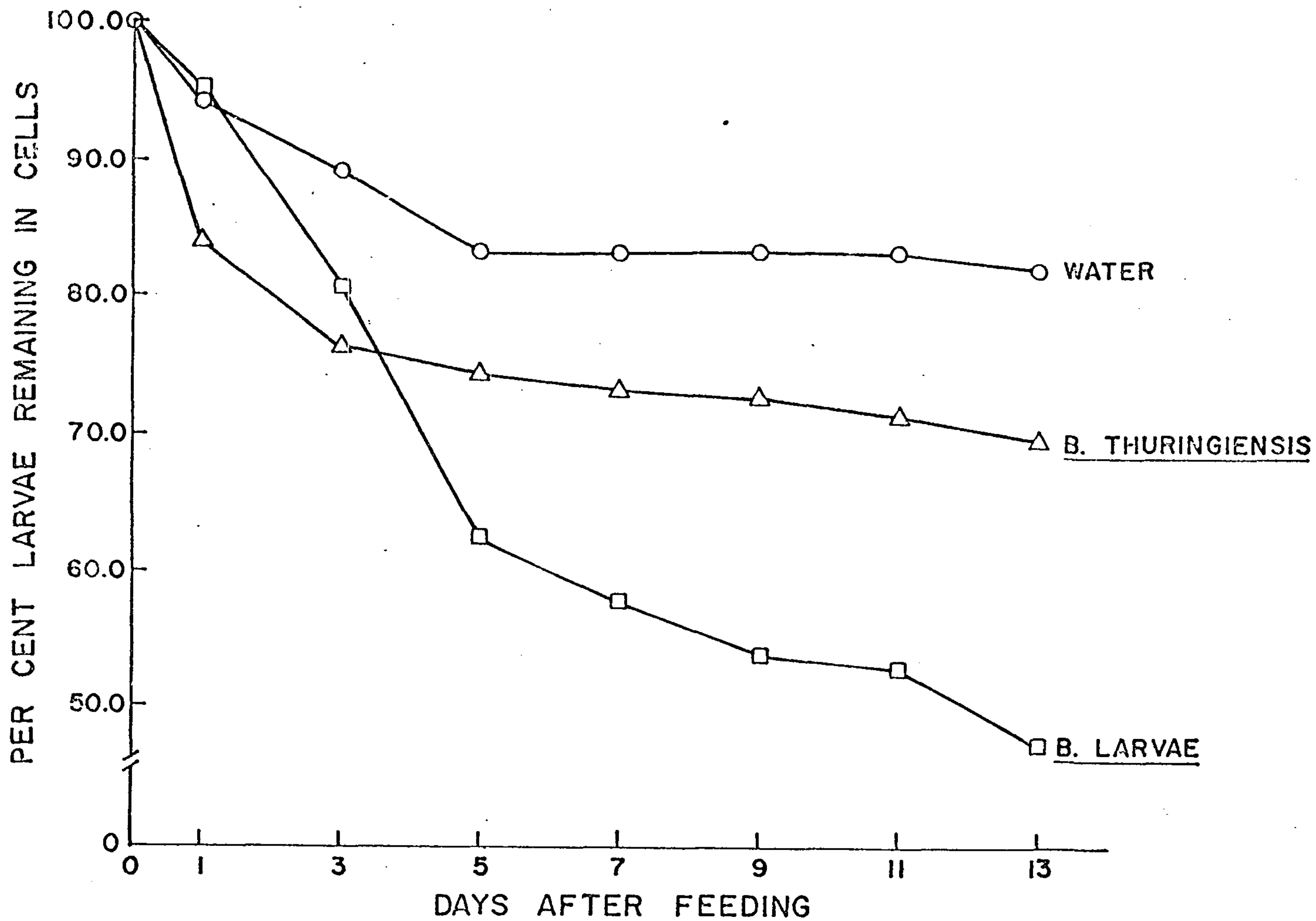

REMOVAL RATE OF 16 HR BROWN LINE LARVAE
PER CENT LARVAE REMAINING IN CELLS
100.0
90.0
80.0
70.0
60.0
50.0
0
DAYS AFTER FEEDING
0
1
3
5
7
9
11
13
WATER
B. THURINGIENSIS
B. LARVAE

Figure 9. Removal rate of 24 hour Brown line larvae

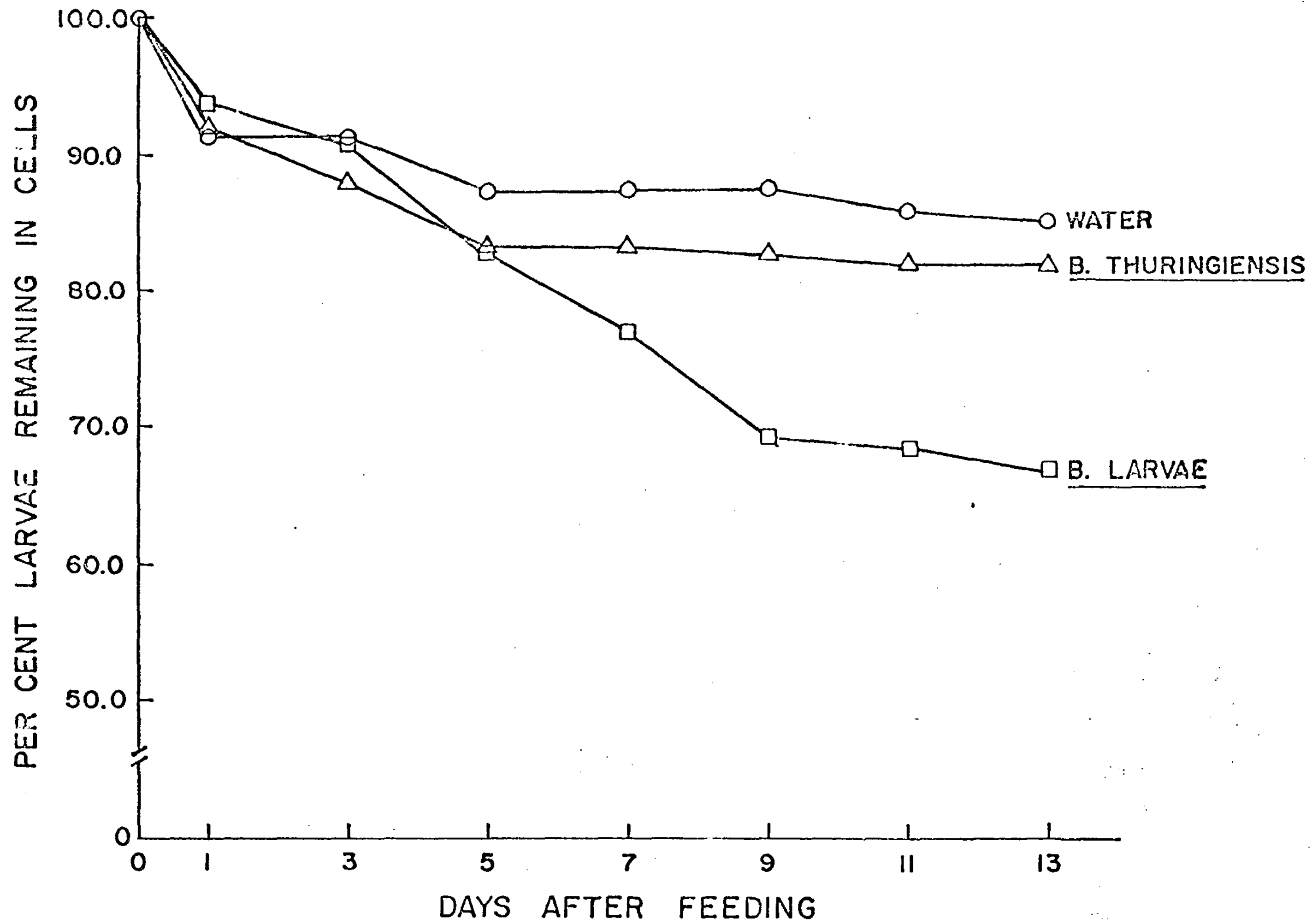
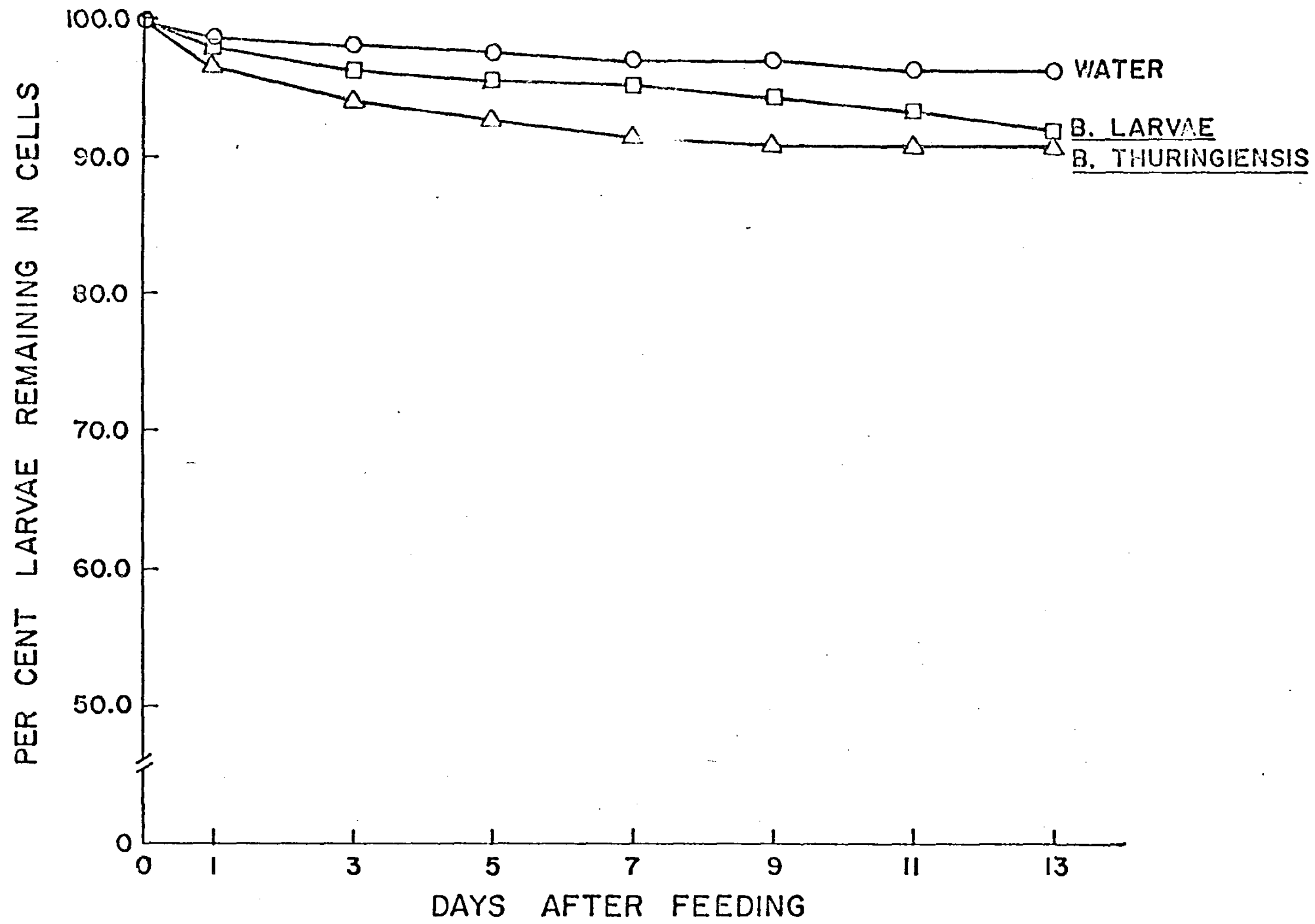

REMOVAL RATE OF 24 HR BROWN LINE LARVAE
PER CENT LARVAE REMAINING IN CELLS
100.0
90.0
80.0
70.0
60.0
50.0
0
0
1
3
5
7
9
11
13
DAYS AFTER FEEDING
WATER
B. THURINGIENSIS
B. LARVAE

Figure 10. Removal rate of 32 hour Brown line larvae


REMOVAL RATE OF 32 HR BROWN LINE LARVAE
PER CENT LARVAE REMAINING IN CELLS
100.0
90.0
80.0
70.0
60.0
50.0
0
0
1
3
5
7
9
11
13
DAYS AFTER FEEDING
WATER
B. LARVAE
B. THURINGIENSIS

Appendix Table 34. Removal rates on days one, five and thirteen were statistically analyzed. No difference in removal rates in the 16, 24, and 32 hour Brown line larvae was found to be significant one day after feeding (Table 29a). However, there does seem to be an apparent difference in the 16 hour Brown line larvae due to feeding B. thuringiensis (Figure 8). About 14 per cent of the 16 hour Brown line larvae was removed from the cells one day after feeding spores of B. thuringiensis. Similarly treated 24 and 32 hour Brown line larvae showed about seven per cent and three per cent removal, respectively, after one day (Figures 9 and 10). There were essentially no differences between water and B. larvae treated brood one day after feeding in 16, 24, and 32 hour Brown line larvae. Even five days after feeding, there were still no statistically significant differences between removal rates of 16, 24, and 32 hour Brown line larvae fed water, B. thuringiensis, or B. larvae (Table 29b). In the 16 and 24 hour Brown line larvae, five day counts, the removal of larvae fed water or B. thuringiensis began to level off; meanwhile, brood fed B. larvae were still being removed (Figures 8 and 9). Differences among the removal rates of all three treatments in the 32 hour Brown line larvae, five days after feeding, were less than 10 per cent (Figure 10). On day 13 the difference in removal due to feeding B. larvae was signifi-

Table 29a. Analyses of variance of Brown line larvae removal rates one day after feeding

Age in hours	Source of variation	Degrees of freedom	Sum of squares	Mean square	F
16	Treatments	2	0.46	0.23	2.09
	Error	9	1.02	0.11	
24	Treatments	2	0.05	0.02	0.12
	Error	6	1.03	0.17	
32	Treatments	2	0.01	0.005	0.08
	Error	6	0.36	0.06	

Table 29b. Analyses of variance of Brown line larvae removal rates five days after feeding

Age in hours	Source of variation	Degrees of freedom	Sum of squares	Mean square	F
16	Treatments	2	4.17	2.08	2.10
	Error	9	8.95	0.99	
24	Treatments	2	0.24	0.12	0.39
	Error	6	1.87	0.31	
32	Treatments	2	0.07	0.04	0.57
	Error	6	0.44	0.07	

Table 29c. Analyses of variance of Brown line larvae removal rates 13 days after feeding

Age in hours	Source of variation	Degrees of freedom	Sum of squares	Mean square	F
16	Treatments	2	12.34	6.17	8.23**
	BT + water versus *B. larvae*	(1)	(11.04)	11.04	14.72**
	BT versus water	(1)	(1.30)	1.30	1.73
	Error	9	6.72	0.75	
24	Treatments	2	2.00	1.00	0.21
	Error	6	2.85	0.48	
32	Treatments	2	0.11	0.06	0.75
	Error	6	0.51	0.08	

cantly greater than larvae which were fed water or *B. thuringiensis* (Table 29c). However, there was no significant difference in removal between 16 hour larvae fed *B. thuringiensis* or water. Differences in the 24 and 32 hour Brown line larvae were all nonsignificant 13 days after feeding (Table 29c). The differences due to age in per cent removal can be seen 13 days after feeding (Figures 8, 9, and 10). Removals due to feeding in 16, 24, and 32 hour Brown line larvae were 17, 13, and three per cent, respec-

tively (Figures 8, 9, and 10). Likewise, a decrease due to age in removal rates for brood fed *B. thuringiensis* or *B. larvae* could be seen.

Figures 11, 12, and 13 show the removal rates of 16, 24, and 32 hour Van Scoy line larvae fed water, *B. thuringiensis*, or *B. larvae*. About 20 per cent of the 16 hour Van Scoy line larvae fed *B. thuringiensis* was removed from the combs (Figure 11). Whereas, in the 16 hour Van Scoy line larvae fed *B. larvae* or water, the removals were six and three per cent, respectively, in one day. The difference in removal rates between brood fed *B. thuringiensis* or water was significant one day after feeding (Table 30a). The difference in removal due to feeding *B. thuringiensis* or water was likewise significant in the 24 hour Van Scoy line larvae one day after feeding (Figure 12 and Table 30a). There were no significant differences in removals due to the treatments in the 32 hour Van Scoy line larvae (Figure 13 and Table 30a).

Even on the fifth day after feeding, the difference in removal between brood fed water or *B. thuringiensis* in the 16 hour Van Scoy larvae was still highly significant (Figure 11 and Table 30b). However, the difference in removal due to AFB (*B. larvae*) was not significantly different from water and *B. thuringiensis* treated larvae. Although in the 24 hour Van Scoy larvae there was an apparent differ-

ence in removals on the fifth day between water and *B. thuringiensis* (Figure 12), this difference was not statistically significant (Table 30b). Similarly, no significant differences were found in the 32 hour Van Scoy larvae among all treatments five days after feeding (Figure 13 and Table 30b).

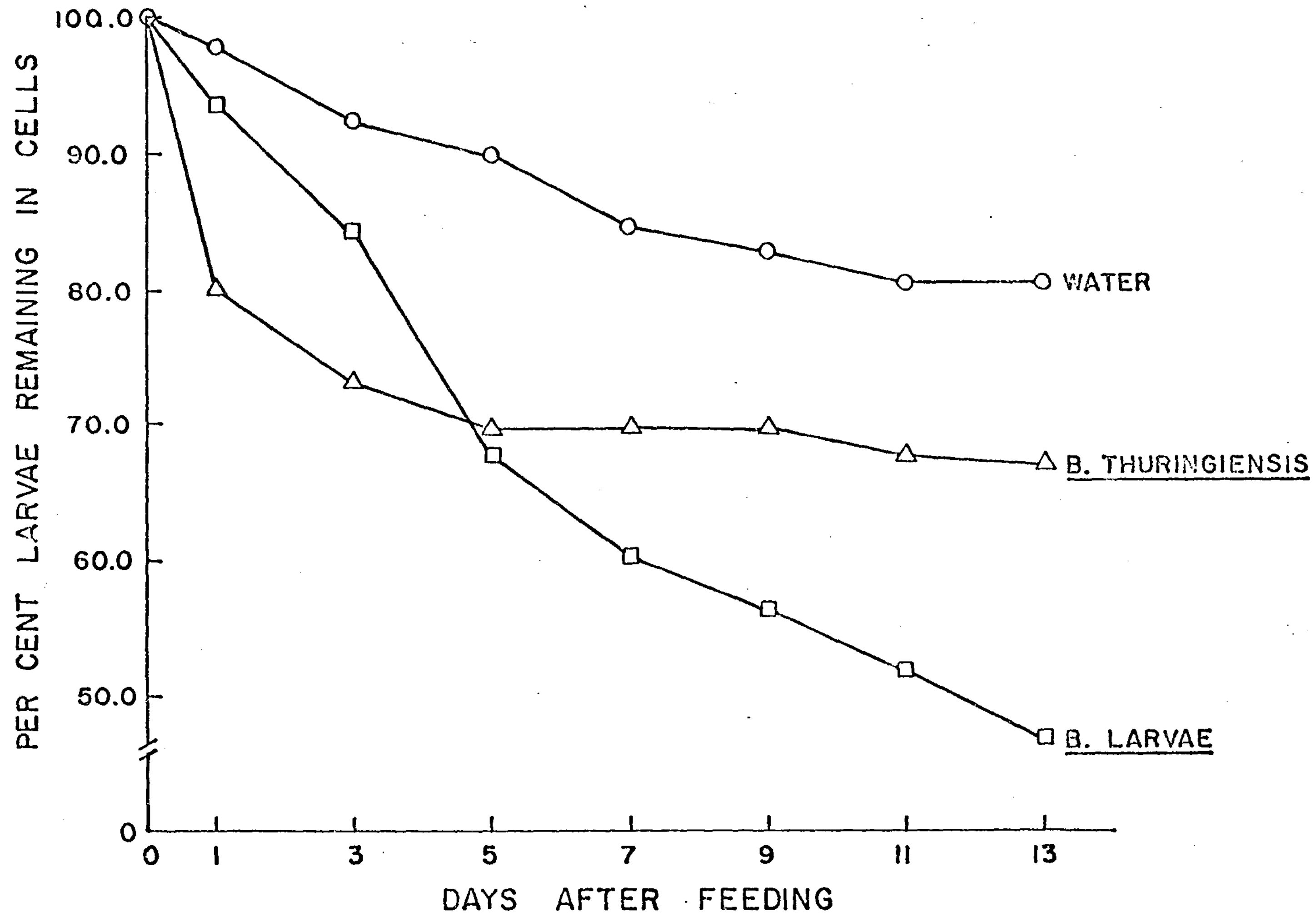
On day 13, there was still a significant difference in removal of larvae fed water or *B. thuringiensis* in 16 hour Van Scoy larvae (Table 30c). There were apparent differences in 24 and 32 hour Van Scoy larvae (Figures 12 and 13), but none of these differences was statistically significant (Table 30c). The difference in removal between AFB and non-AFB (*B. thuringiensis*) and water was significant in only 16 and 24 hour Van Scoy line larvae (Table 30c). The differences in removal due to age are not as striking as in the Brown line larvae; however, the Van Scoy line of bees do not become completely immune to AFB until about 45-48 hours, whereas the Brown line of bees become completely resistant at 35 hours (Bamrick, 1960).

The differences in relative "susceptibility" to *B. thuringiensis* could be due to the average weight of the larvae. Sutter (1963) postulated that differences in resistance to AFB of Brown and Van Scoy line larvae could be due to the relatively faster growth rate of the Brown line larvae. Thus, the Brown line larvae showed no statis-

tically significant removal rates due to *B. thuringiensis*, while, in both 16 and 24 hour Van Scoy line larvae, statistically significant removals were caused by *B. thuringiensis*.

There is no apparent explanation for the removal of young larvae fed the *B. thuringiensis* spore suspension. Perhaps the crystals present in the spore suspension, an electron micrograph of which is shown in Figure 7, could cause adverse effects. Hall and Dunn (1958) observed that *Sabulodes caberata* starved to death after feeding on *B. thuringiensis* coated foliage. Possibly, this could also be the case with honey bee larvae. Another possible factor could be the odor of the spore suspension. Nevertheless, until the factor(s) that result in removal of larvae can be eliminated, it would be necessary to exercise caution in the use of *B. thuringiensis* in areas where the worker bees might forage.

Figure 11. Removal of 16 hour Van Scoy larvae



REMOVAL RATE OF 16 HR VAN SCOY LARVAE
PER CENT LARVAE REMAINING IN CELLS
100.0
90.0
80.0
70.0
60.0
50.0
0
0
1
3
5
7
9
11
13
DAYS AFTER FEEDING
WATER
B. THURINGIENSIS
B. LARVAE

Figure 12. Removal of 24 hour Van Scoy larvae

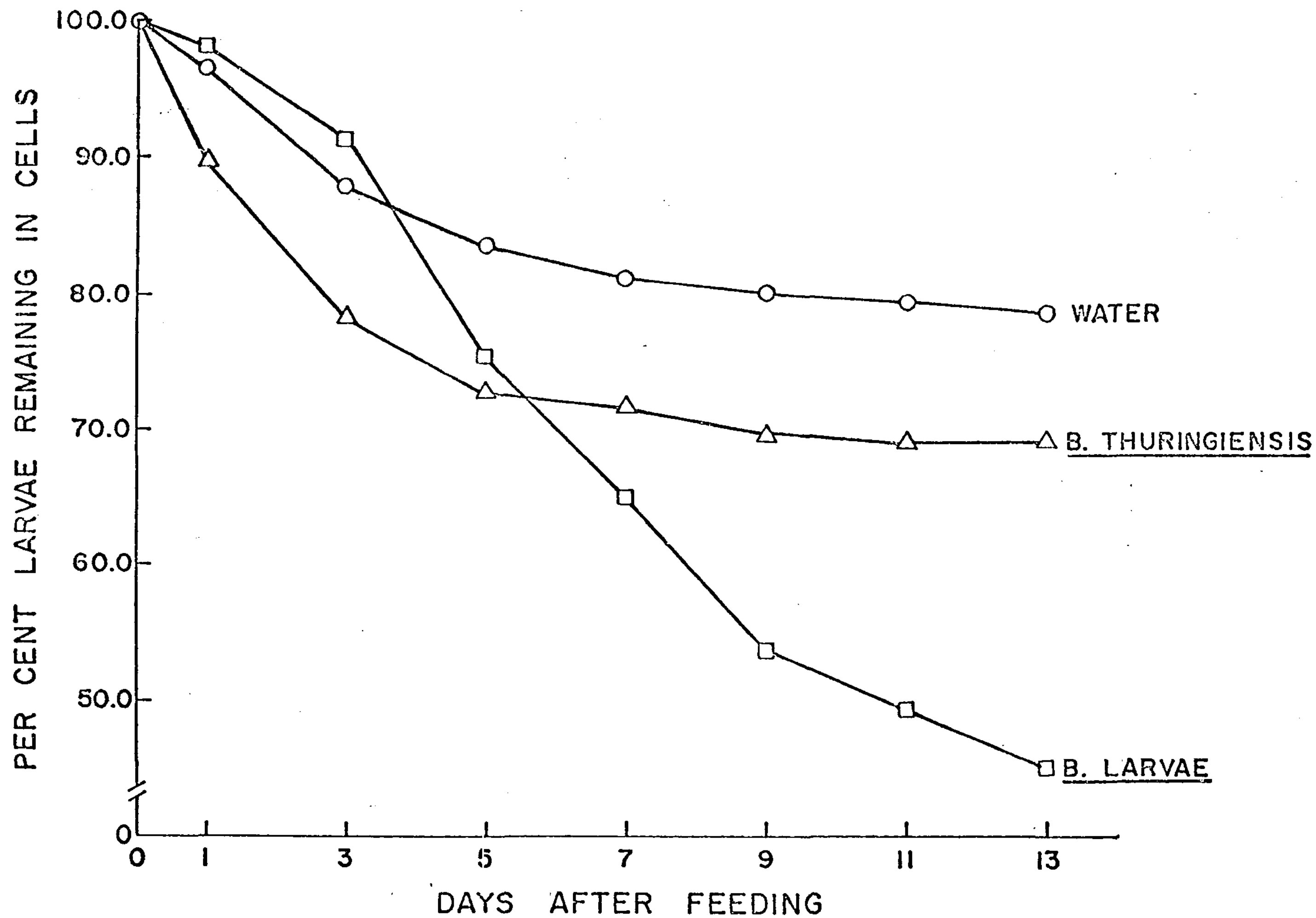

REMOVAL RATE OF 24 HR VAN SCOY LARVAE
PER CENT LARVAE REMAINING IN CELLS
100.0
90.0
80.0
70.0
60.0
50.0
0
0
1
3
5
7
9
11
13
DAYS AFTER FEEDING
WATER
B. THURINGIENSIS
B. LARVAE

Figure 13. Removal of 32 hour Van Scoy larvae

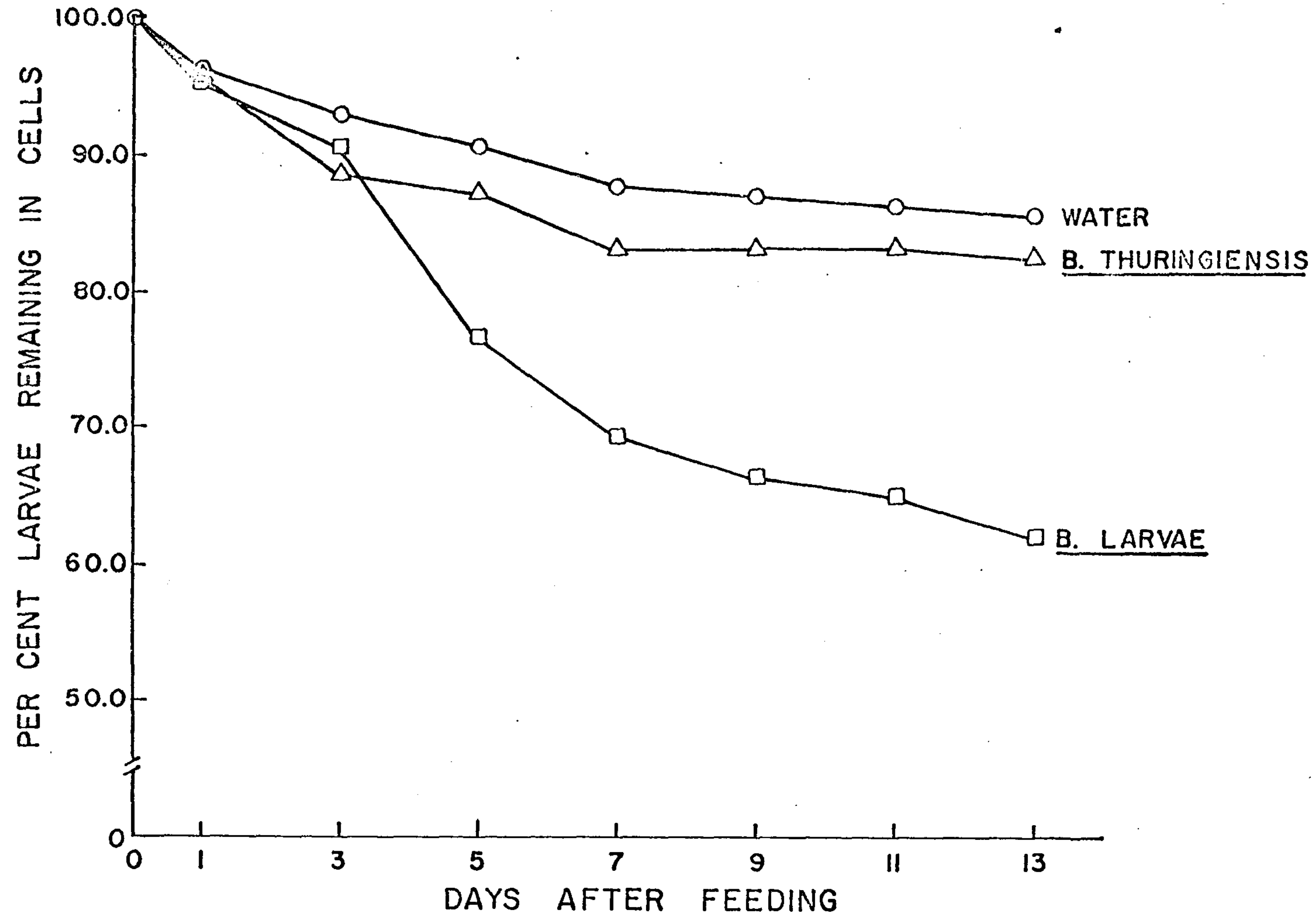

REMOVAL RATE OF 32 HR VAN SCOY LARVAE
PER CENT LARVAE REMAINING IN CELLS
100.0
90.0
80.0
70.0
60.0
50.0
0
0
1
3
5
7
9
11
13
DAYS AFTER FEEDING
WATER
B. THURINGIENSIS
B. LARVAE

Table 30a. Analyses of variance of Van Scoy line larvae removal rates one day after feeding

Age in hours	Source of variation	Degrees of freedom	Sum of squares	Mean square	F
16	Treatments	2	1.79	0.90	22.50**
	BT + water versus *B. larvae*	(1)	0.16	0.16	4.00
	BT versus water	(1)	1.63	1.63	40.75**
	Error	9	0.40	0.04	
24	Treatments	2	0.51	0.26	5.20*
	BT + water versus *B. larvae*	(1)	(0.18)	0.18	3.60
	BT versus water	(1)	(0.33)	0.33	6.60*
	Error	12	0.65	0.05	
32	Treatments	2	0.01	0.005	0.86
	Error	11	0.47	0.043	

Table 30b. Analyses of variance of Van Scoy line larvae removal rates five days after feeding

Age in hours	Source of variation	Degrees of freedom	Sum of squares	Mean square	F
16	Treatments	2	3.64	1.82	7.28*
	BT + water versus *B. larvae*	(1)	(1.16)	1.16	4.64
	BT versus water	(1)	(2.48)	2.48	9.92*
	Error	9	2.22	0.25	
24	Treatments	2	1.26	0.63	0.38
	Error	12	19.87	1.66	
32	Treatments	2	1.22	0.61	1.11
	Error	9	4.14	0.46	

Table 30c. Analyses of variance of Van Scoy line larvae removal rates 13 days after feeding

Age in hours	Source of variation	Degrees of freedom	Sum of square	Mean square	F
16	Treatments	2	8.85	4.42	16.37**
	BT + water versus *B. larvae*	(1)	(7.66)	7.66	28.37**
	BT versus water	(1)	(1.19)	4.41	16.33**
	Error	9	2.42	0.27	
24	Treatments	2	11.39	5.70	4.38*
	BT + water versus *B. larvae*	(1)	(10.20)	10.20	7.85*
	BT versus water	(1)	(1.19)	1.19	0.92
	Error	12	15.60	1.30	
32	Treatments	2	4.53	2.26	3.90
	Error	9	5.23	0.58	

SUMMARY

Optimum conditions for growth of Bacillus larvae in vitro were found to be pH 6.5 - 7.5 at 31 - 40° centigrade. A medium (medium E) of 1.0 per cent Difco Yeast Extract and 1.0 per cent soluble starch was as satisfactory as other more complex media for the cultivation of B. larvae in vitro. The presence of agar in medium B resulted in a banding of growth at levels determined by the amount of agar present in the medium. Glucose autoclaved with agar exerted a detrimental effect on the growth of B. larvae.

A number of organic nitrogen sources were tested for their ability to support growth of B. larvae. Proteose Peptone No. 3 and Difco Yeast Extract were the most promising of the medium constituents tested. Brand-to-brand differences in the ability of yeast extract to support growth of B. larvae were found; however, no difference in batches from one producer was found.

The Most Probable Numbers method was impractical for the enumeration of B. larvae spores because of the prolonged incubation periods required to obtain maximum growth. Viable counts of B. larvae were shown to be feasible. Media BA and EA were found to yield the most satisfactory results. Although colonies of B. larvae were small, the use of 2, 3, 5 triphenyltetrazolium chloride enabled plate counts to be

successfully made.

Significant differences were shown in virulence between various strains of *B. larvae*. *In vitro* prepared spore suspensions of *B. larvae* were as virulent as *in vivo* spores. Differences in virulence among spores produced *in vitro* were also demonstrated. Virulence of *B. larvae* spores was shown to be increased by even one passage in either resistant or susceptible lines of honey bees. The age of larvae, at the time of inoculation, did not significantly alter the virulence of *B. larvae*, except in one instance which was believed to be due to experimental error.

Bacillus thuringiensis spores and crystals in the larval food of honey bee larvae did not cause disease symptoms; however, an abnormally high rate of larval removal, one day after feeding, was demonstrated. This removal was statistically significant in only 16 and 24 hour Van Scoy (AFB susceptible) line larvae. The removal in Brown (AFB resistant) line larvae of 16 and 24 hours was not significant.

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APPENDIX

Table 31a. Sample statistical analyses

Queen mating number	Per cent nonsurvival[a]		
	Water	Sturtevant	SFVV
M1839	2.08	9.34	9.87
	2.85	9.66	9.92
	2.31	9.41	10.05
	2.90	9.48	10.05
M1840	1.00	8.29	9.59
	3.05	9.00	9.48
	2.62	8.46	9.87

Total: $\Sigma X = 149.28 \quad \Sigma X^2 = 1300.73$

Treatment sums: Water, 16.81; Sturtevant, 63.64; SFVV, 68.83

Queen sums: M1839, 87.92; M1840, 61.36

Correction: $C = \dfrac{(149.28)^2}{21} = 1061.17$

Total: $\Sigma X^2 - C = 1300.73 - 1061.17 = 239.56$

Sub-classes: $\dfrac{(10.14)^2 + (37.89)^2 + (39.89)^2}{4}$

$+ \dfrac{(6.67)^2 + (25.75)^2 + (28.94)^2}{3} - C = 236.28$

Error: $239.56 - 236.28 = 3.28$

Treatments: $\dfrac{(16.81)^2 + (63.64)^2 + (68.83)^2}{7} - C = 234.57$

[a]Nonsurvival percentages transformed to $\sqrt{X + 1}$.

Table 31a. (Continued)

Queens: $\frac{(87.92)^2 + (61.36)^2}{9} - C = 1.33$

Interaction: $236.28 - (234.57 + 1.33) = 0.38$

Water versus spores: $\frac{[-2(16.81) + 63.64 + 68.83]^2}{(6)(7)} = 232.65$

Sturtevant versus SFVV: $\frac{(63.64 - 68.83)^2}{(2)(7)} = 1.92$

Table 31b. Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	2	234.57	117.28	533.09**
Water versus spores	(1)	(232.65)	232.65	1057.50**
Sturtevant versus SFVV	(1)	(1.92)	1.92	8.73*
Queens	1	1.33	1.33	6.04*
Interaction	2	0.38	0.19	0.86
Error	15	3.28	0.22	
Total	20	239.56		

Table 32. Raw data of *in vivo* studies of *B. larvae*

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1773	16	Brown	Water	23	22	1	0	4.35
			"X"	16	1	15	0	93.75
			Ames	17	0	14	3	100.00
M1773	16	Brown	Water	36	36	0	0	0.00
			"X"	43	3	26	14	93.02
			Ames	44	4	21	19	90.91
M1962	24	Van Scoy	Water	37	35	2	0	5.40
			1c	50	30	11	9	40.00
			2c	46	38	7	1	17.39
M2087	24	Van Scoy	Water	24	24	0	0	0.00
			1c	22	5	13	4	77.27
			2c	25	18	4	3	28.00
M1962	24	Van Scoy	Water	46	43	3	0	6.52
			1c	54	21	17	16	61.11
			2c	48	42	3	3	12.50
M2088	24	Van Scoy	Water	21	20	1	0	4.76
			1c	18	4	9	5	77.78
			2c	19	10	7	2	47.37
M2085[a]	24	Van Scoy	Water	17	14	1	2	17.65
			1c	19	2	8	9	89.47
			2c	18	17	8	7	44.12

[a]Data not included in analyses.

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2083	24	Van Scoy	Water	17	17	0	0	0.00
			1c	16	3	4	9	81.25
			2c	18	16	1	1	11.11
M2084	24	Van Scoy	Water	23	22	1	0	4.35
			1c	26	7	11	8	73.33
			2c	28	22	2	4	22.43
M2083	24	Van Scoy	Water	32	30	2	0	6.25
			1c	40	16	3	21	60.00
			2c	28	13	2	13	53.57
M2084	24	Van Scoy	Water	33	31	2	0	6.06
			1c	35	5	12	18	85.71
			2c	38	27	0	11	28.95
M2089[a]	24	Van Scoy	Water	28	19	3	6	32.14
			1c	25	6	5	14	76.00
			2c	32	18	4	10	43.75
M2083	24	Van Scoy	Water	26	26	0	0	0.00
			1a	25	17	5	3	32.00
			2b	31	20	8	3	32.26
M2083	24	Van Scoy	Water	22	22	0	0	0.00
			1a	27	17	7	3	37.04
			2b	23	16	5	2	30.43

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2083[a]	24	Van Scoy	Water	18	16	0	2	11.11
			1a	17	8	3	6	52.94
			2b	17	10	2	5	41.18
M2083[a]	24	Van Scoy	Water	39	34	2	2	12.82
			1a	28	13	3	12	53.57
			2b	27	12	2	13	55.56
M2083	24	Van Scoy	Water	27	26	1	0	3.70
			1a	27	20	2	5	25.92
			2b	20	15	4	1	25.00
M2083	24	Van Scoy	Water	31	31	0	0	0.00
			1a	30	13	10	7	56.67
			2b	24	10	5	9	58.33
M2085	24	Van Scoy	Water	12	12	0	0	0.00
			1a	20	12	2	6	40.00
			2b	19	5	7	7	73.68
M2085[a]	24	Van Scoy	Water	17	15	2	0	11.76
			1a	22	7	5	10	68.18
			2b	22	7	10	5	68.18
M2092	24	Van Scoy	Water	33	33	0	0	0.00
			1a	47	36	4	7	23.40
			2b	38	20	6	12	47.37

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2089	24	Van Scoy	Water	17	17	0	0	0.00
			1a	24	17	3	4	29.17
			2b	29	11	11	7	62.07
M2092	24	Van Scoy	Water	41	40	1	0	2.44
			1a	46	28	6	12	39.13
			2b	56	20	21	15	64.29
M2089	24	Van Scoy	Water	36	34	1	1	5.55
			1a	39	15	4	20	61.54
			2b	35	9	10	16	74.28
M2094	24	Van Scoy	Water	23	23	0	0	0.00
			1a	27	10	12	5	62.96
			2b	27	11	10	6	59.26
M1773	16	Brown	Water	54	51	3	0	5.56
			SF	48	27	17	4	43.75
			Sturtevant	48	11	11	26	47.08
M1784[a]	16	Brown	Water	30	26	3	1	13.33
			SF	33	7	17	9	78.79
			Sturtevant	28	8	10	10	71.43
M1760[a]	24	Van Scoy	Water	52	41	11	0	21.15
			SF	42	30	10	2	28.57
			Sturtevant	53	37	10	6	30.19

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1763	24	Van	Water	48	44	3	1	8.33
		Scoy	SF	40	23	3	14	42.50
			Sturtevant	44	15	10	19	65.91
M1840[a]	16	Van	Water	31	27	4	0	12.93
		Scoy	SFV	39	0	28	11	100.00
			SFB	39	2	29	8	94.87
M1840	16	Van	Water	25	23	2	0	8.00
		Scoy	SFV	34	1	27	6	97.06
			SFB	29	2	20	7	93.10
M1840	16	Van	Water	28	26	2	0	7.14
		Scoy	SFV	33	4	12	17	87.88
			SFB	36	4	15	17	88.89
M1839	16	Van	Water	37	36	0	1	2.77
		Scoy	SFV	36	0	21	15	100.00
			SFB	42	1	24	17	97.61
M1840[a]	24	Van	Water	42	36	6	0	14.28
		Scoy	SFV	34	2	23	9	94.11
			SFB	39	5	23	11	87.20
M1804[a]	24	Van	Water	21	18	3	0	14.28
		Scoy	SFV	19	3	13	3	84.21
			SFB	20	4	15	1	80.00

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1840	24	Van Scoy	Water	42	42	0	0	0.00
			SFV	36	5	15	16	86.11
			SFB	44	2	21	21	95.45
M1874	16	Brown	Water	38	36	2	0	5.26
			SFV	37	0	36	1	100.00
			SFB	35	0	22	13	100.00
M1874	16	Brown	Water	37	33	4	0	10.81
			SFV	48	3	42	3	93.75
			SFB	32	1	21	10	96.87
M1799	16	Brown	Water	40	37	2	1	7.50
			SFV	52	3	22	27	94.23
			SFB	52	2	14	36	96.15
M1799	16	Brown	Water	26	25	1	0	3.84
			SFV	44	2	35	7	95.45
			SFB	33	3	23	7	90.91
M1799	24	Brown	Water	74	69	5	0	6.76
			SFV	74	9	30	35	87.83
			SFB	104	21	33	50	79.87
M1799	24	Brown	Water	21	19	2	0	9.52
			SFV	14	0	8	6	100.00
			SFB	8	0	2	6	100.00

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1799	16	Brown	Water	68	62	6	0	8.82
			SFVV	70	0	26	44	100.00
			SFBB	71	2	31	38	97.18
M1799	16	Brown	Water	80	76	4	0	5.00
			SFVV	79	8	25	46	89.87
			SFBB	84	9	23	52	89.28
M1799	24	Brown	Water	72	67	4	1	6.94
			SFVV	60	14	13	33	76.67
			SFBB	77	18	15	44	76.62
M1799	24	Brown	Water	31	29	2	0	6.45
			SFVV	21	11	5	5	47.61
			SFBB	29	9	6	14	68.27
M1799	24	Brown	Water	37	34	3	0	8.11
			SFVV	41	4	22	15	90.24
			SFBB	43	6	17	20	86.05
M1839	16	Van Scoy	Water	23	22	1	0	4.35
			Sturtevant	32	4	12	16	87.50
			SFVV	38	0	26	12	100.00
M1840[a]	16	Van Scoy	Water	31	23	9	0	25.86
			Sturtevant	46	17	11	18	63.17
			SFVV	30	1	17	12	96.67

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1839	16	Van	Water	28	26	2	0	7.14
		Scoy	Sturtevant	39	3	22	14	92.30
			SFVV	38	1	22	15	97.36
M1839	16	Van	Water	30	29	1	0	3.33
		Scoy	Sturtevant	29	4	13	12	86.20
			SFVV	28	1	19	8	96.42
M1840	16	Van	Water	33	33	0	0	0.00
		Scoy	Sturtevant	31	10	8	13	67.74
			SFVV	33	3	16	14	90.91
M1840	16	Van	Water	34	32	1	1	5.88
		Scoy	Sturtevant	34	10	11	13	70.58
			SFVV	28	1	19	8	96.42
M1840	16	Van	Water	36	33	3	0	8.33
		Scoy	Sturtevant	25	5	6	14	80.00
			SFVV	27	3	20	4	88.89
M1839	16	Van	Water	27	25	2	0	7.40
		Scoy	Sturtevant	27	3	13	11	88.89
			SFVV	22	0	18	4	100.00
M1840	24	Van	Water	20	0	0	1	5.00
		Scoy	Sturtevant	20	9	4	7	55.00
			SFVV	22	4	13	5	81.82

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1839	24	Van Scoy	Water	23	21	2	0	8.69
			Sturtevant	32	13	9	10	59.37
			SFVV	36	2	26	8	94.44
M1840	24	Van Scoy	Water	26	24	2	0	7.69
			Sturtevant	24	11	8	5	54.16
			SFVV	32	5	14	13	84.37
M1839	24	Van Scoy	Water	22	20	1	1	9.09
			Sturtevant	16	7	7	2	56.25
			SFVV	18	1	13	4	94.44
M1839	24	Van Scoy	Water	18	17	1	0	5.55
			Sturtevant	19	6	7	6	68.42
			SFVV	18	0	8	10	100.00
M1799	16	Brown	Water	63	59	4	0	6.34
			Sturtevant	51	12	17	22	76.47
			SFVV	57	3	33	21	94.73
M1799	16	Brown	Water	62	57	0	5	8.06
			Sturtevant	59	19	12	28	67.79
			SFVV	53	5	25	23	90.56
M1874	16	Brown	Water	21	20	1	0	4.76
			Sturtevant	21	6	4	11	71.42
			SFVV	24	2	10	12	91.67

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1799	24	Brown	Water	64	60	3	1	6.25
			Sturtevant	45	21	8	16	53.33
			SFVV	57	2	23	32	96.49
M1874[a]	24	Brown	Water	18	13	5	0	27.78
			Sturtevant	20	1	11	8	95.00
			SFVV	24	0	15	9	100.00
M1799	24	Brown	Water	30	28	2	0	6.67
			Sturtevant	45	27	10	8	40.00
			SFVV	36	4	2	20	88.89
M1799	24	Brown	Water	42	39	3	0	7.14
			Sturtevant	49	27	7	15	44.89
			SFVV	44	2	9	33	95.45
M1840	16	Van Scoy	Water	41	38	3	0	7.31
			Sturtevant	38	13	12	13	65.79
			SFBB	29	1	22	6	96.55
M1839	16	Van Scoy	Water	33	32	1	0	3.33
			Sturtevant	32	5	17	10	84.37
			SFBB	32	0	30	2	100.00
M1840	16	Van Scoy	Water	44	41	2	1	6.81
			Sturtevant	43	11	16	16	74.41
			SFBB	35	2	21	12	94.28

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1840	16	Van Scoy	Water	30	27	3	0	10.00
			Sturtevant	24	3	6	15	87.50
			SFBB	30	1	18	11	96.67
M1839[a]	16	Van Scoy	Water	16	13	3	0	18.75
			Sturtevant	18	3	2	13	83.33
			SFBB	14	0	9	5	100.00
M1840[a]	24	Van Scoy	Water	23	20	2	1	13.04
			Sturtevant	23	10	5	8	56.52
			SFBB	19	0	10	9	100.00
M1839	24	Van Scoy	Water	28	27	0	1	3.57
			Sturtevant	35	11	10	14	68.57
			SFBB	46	1	36	9	97.82
M1840[a]	24	Van Scoy	Water	24	21	1	2	12.50
			Sturtevant	23	16	3	4	30.43
			SFBB	23	4	6	13	82.60
M1840	24	Van Scoy	Water	38	35	3	0	7.89
			Sturtevant	27	11	3	13	59.25
			SFBB	33	1	16	16	96.97
M1840	24	Van Scoy	Water	26	26	0	0	0.00
			Sturtevant	40	10	7	23	75.00
			SFBB	35	2	19	14	94.28

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M1839	24	Van Scoy	Water	32	29	3	0	9.37
			Sturtevant	22	6	5	11	72.72
			SFBB	30	1	12	17	96.67
M1799[a]	16	Brown	Water	75	62	12	1	17.33
			Sturtevant	79	13	46	20	83.54
			SFBB	73	1	61	11	98.63
M1799	16	Brown	Water	58	54	4	0	6.89
			Sturtevant	58	17	13	28	70.68
			SFBB	56	5	23	28	91.07
M1799	16	Brown	Water	79	75	3	1	5.06
			Sturtevant	82	31	13	38	62.19
			SFBB	81	0	35	46	100.00
M1799	24	Brown	Water	63	57	3	3	9.52
			Sturtevant	71	47	15	9	35.21
			SFBB	72	8	43	21	88.89
M1799	24	Brown	Water	35	33	2	0	5.71
			Sturtevant	58	25	17	16	56.89
			SFBB	49	5	20	24	89.79
M1799	24	Brown	Water	56	51	5	0	8.92
			Sturtevant	58	37	7	14	36.20
			SFBB	64	8	16	40	87.50

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2084	6	Van	Water	19	19	0	0	0.00
		Scoy	Sturtevant	35	4	20	11	88.57
M2085	6	Van	Water	17	17	0	0	0.00
		Scoy	Sturtevant	31	2	14	15	93.55
M2085	6	Van	Water	10	9	1	0	10.00
		Scoy	Sturtevant	27	4	18	5	85.18
M2083[a]	6	Van	Water	13	10	0	3	23.08
		Scoy	Sturtevant	15	1	13	1	93.33
M2083	6	Van	Water	12	11	0	1	8.33
		Scoy	Sturtevant	22	4	14	4	81.82
M2083	40	Van	Water	29	29	0	0	0.00
		Scoy	Sturtevant	51	47	4	0	7.74
M2084	40	Van	Water	13	12	1	0	7.69
		Scoy	Sturtevant	39	29	6	4	25.64
M2084	40	Van	Water	24	24	0	0	0.00
		Scoy	Sturtevant	48	42	3	3	12.50
M2085	40	Van	Water	15	15	0	0	0.00
		Scoy	Sturtevant	50	43	1	6	14.00
M2084	40	Van	Water	18	17	1	0	5.55
		Scoy	Sturtevant	44	34	3	7	22.73

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2085	40	Van	Water	22	21	0	1	4.54
		Scoy	Sturtevant	48	42	1	5	12.50
M2088[a]	40	Van	Water	17	12	4	1	29.41
		Scoy	Sturtevant	35	24	9	2	31.43
M2083	40	Van	Water	21	21	0	0	0.00
		Scoy	Sturtevant	45	45	0	0	0.00
M2084	40	Van	Water	26	24	0	2	7.69
		Scoy	Sturtevant	41	34	2	5	17.07
M2084	6	Van	Water	28	26	2	0	7.14
		Scoy	SY-1	70	7	41	22	90.00
M2087	6	Van	Water	21	19	0	2	9.52
		Scoy	SY-1	40	3	15	22	92.50
M2083	6	Van	Water	33	32	0	1	3.33
		Scoy	SY-1	71	2	34	35	97.17
M1962	6	Van	Water	20	20	0	0	0.00
		Scoy	SY-1	40	7	10	23	82.50
M1959[a]	6	Van	Water	25	15	10	0	40.00
		Scoy	SY-1	45	0	30	15	100.00
M2085	6	Van	Water	28	26	1	1	7.14
		Scoy	SY-1	67	3	37	27	95.52

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2084	40	Van	Water	50	50	0	0	0.00
		Scoy	SO-1	90	82	4	4	8.89
M2087	40	Van	Water	42	41	0	1	2.38
		Scoy	SO-1	71	67	1	3	5.63
M2083	40	Van	Water	35	35	0	0	0.00
		Scoy	SO-1	91	81	3	7	10.99
M2088	40	Van	Water	29	28	1	0	3.45
		Scoy	SO-1	55	51	1	3	7.27
M1959	40	Van	Water	20	20	0	0	0.00
		Scoy	SO-1	40	36	1	3	10.00
M2083	40	Van	Water	40	40	0	0	0.00
		Scoy	SO-1	99	95	2	2	4.04
M2083	24	Van	Water	31	31	0	0	0.00
		Scoy	SY-2L	41	14	2	25	65.85
			SY-2P	27	3	5	19	88.89
M2084	24	Van	Water	45	42	3	0	6.67
		Scoy	SY-2L	41	12	5	24	70.73
			SY-2P	45	7	9	29	84.44
M2084	24	Van	Water	32	29	2	1	9.38
		Scoy	SY-2L	43	9	8	26	79.07
			SY-2P	42	3	7	32	92.86

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2087	24	Van	Water	34	32	2	0	6.25
		Scoy	SY-2L	32	3	6	23	90.62
			SY-2P	37	2	13	22	94.59
M2087	24	Van	Water	32	29	2	1	9.38
		Scoy	SY-2L	29	4	12	13	86.21
			SY-2P	37	1	17	19	97.30
M2084	24	Van	Water	40	38	2	0	5.00
		Scoy	SY-2L	45	5	12	28	88.89
			SY-2P	39	5	4	30	87.18
M2088	24	Van	Water	21	19	2	0	9.52
		Scoy	SO-2L	23	3	11	9	86.96
			SO-2P	22	1	10	11	95.45
M2088	24	Van	Water	21	20	1	0	4.76
		Scoy	SO-2L	30	3	18	9	90.00
			SO-2P	32	2	8	22	93.75
M2083	24	Van	Water	27	25	2	0	7.41
		Scoy	SO-2L	27	5	15	7	81.48
			SO-2P	30	7	17	6	76.67
M2088	24	Van	Water	25	24	0	1	4.00
		Scoy	SO-2L	35	1	12	22	97.14
			SO-2P	34	2	11	21	94.12

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2083	24	Van Scoy	Water	36	33	3	0	8.33
			SO-2L	36	5	12	19	86.11
			SO-2P	37	2	17	18	94.59
M2087	24	Van Scoy	Water	24	22	2	0	8.33
			SO-2L	28	2	11	15	92.86
			SO-2P	28	2	8	18	92.86
M2084	24	Van Scoy	Water	29	27	2	0	6.90
			SO-2L	32	5	7	20	84.38
			SO-2P	34	3	9	22	91.18
M2083	24	Van Scoy	Water	37	34	2	1	8.11
			SO-2L	42	6	17	19	85.71
			SO-2P	35	1	13	21	97.14
M2087	24	Van Scoy	Water	31	30	1	0	3.22
			SO-2L	32	5	10	17	84.38
			SO-2P	46	13	13	20	71.74
M2084	24	Van Scoy	Water	45	43	2	0	4.44
			SO-2L	47	7	7	33	85.11
			SO-2P	52	12	13	27	76.92
M2088	24	Van Scoy	Water	38	38	0	0	0.00
			SO-2L	43	8	7	28	81.40
			SO-2P	43	5	7	31	88.37

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2084	24	Van	Water	46	42	4	0	8.70
		Scoy	SO-2L	36	7	7	22	80.56
			SO-2P	42	4	10	28	90.48
M2088	24	Van	Water	47	45	2	0	4.26
		Scoy	SO-2L	45	11	11	23	75.56
			SO-2P	50	10	10	30	80.00
M2087	24	Van	Water	45	41	4	0	8.89
		Scoy	SO-2L	41	5	8	28	87.80
			SO-2P	43	5	14	24	81.40
M2084	24	Van	Water	51	50	1	0	1.96
		Scoy	SO-2L	42	7	11	24	83.33
			SO-2P	38	5	15	18	86.84
M2084	24	Van	Water	18	17	0	1	5.56
		Scoy	Sturtevant	20	9	2	9	55.00
			Ames	16	0	8	8	100.00
M2084	24	Van	Water	15	14	1	0	6.67
		Scoy	Sturtevant	8	2	1	5	75.00
			Ames	16	1	4	11	93.75
M2083	24	Van	Water	21	21	0	0	0.00
		Scoy	Sturtevant	23	7	4	12	69.57
			Ames	25	1	7	17	96.00

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2083	24	Van	Water	22	20	0	2	9.09
		Scoy	Sturtevant	34	19	1	14	44.12
			Ames	21	2	3	16	90.48
M2083	24	Van	Water	29	29	0	0	0.00
		Scoy	Sturtevant	28	14	2	12	50.00
			Ames	13	3	8	2	76.92
M2088	24	Van	Water	14	13	1	0	7.14
		Scoy	Sturtevant	15	9	4	2	40.00
			Ames	18	1	14	3	94.44
M2088	24	Van	Water	14	14	0	0	0.00
		Scoy	Sturtevant	24	21	3	0	12.50
			SV	22	8	3	11	63.64
M2083	24	Van	Water	34	33	1	0	2.94
		Scoy	Sturtevant	27	23	2	2	14.81
			SV	41	9	3	29	78.05
M2084	24	Van	Water	16	15	1	0	6.25
		Scoy	Sturtevant	35	28	4	3	20.00
			SV	20	4	5	11	80.00
M2088	24	Van	Water	24	24	0	0	0.00
		Scoy	Sturtevant	27	25	0	2	7.41
			SV	31	16	7	8	48.39

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2083	24	Van Scoy	Water	44	43	1	0	2.27
			Sturtevant	33	25	4	4	24.24
			SV	37	9	8	20	75.68
M2084	24	Van Scoy	Water	24	23	1	0	4.17
			Sturtevant	27	22	1	4	18.52
			SV	23	10	3	10	56.52
M2087	24	Van Scoy	Water	27	25	1	1	7.41
			Sturtevant	30	23	2	5	23.33
			SV	34	10	3	21	70.59
M2084	24	Van Scoy	Water	25	23	2	0	8.00
			Sturtevant	15	12	1	2	20.00
			SV	16	5	6	5	68.75
M2085	24	Van Scoy	Water	25	23	2	0	8.00
			Sturtevant	34	26	4	4	23.53
			SV	30	16	3	11	46.67
M2083	24	Van Scoy	Water	27	26	1	0	3.70
			Sturtevant	35	27	6	2	22.86
			SV	33	9	10	14	72.73
M2088	24	Van Scoy	Water	35	32	3	0	8.57
			Ames	33	22	4	7	33.33
			AV	40	1	17	22	97.50

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2083	24	Van	Water	29	28	1	0	3.45
		Scoy	Ames	41	16	13	12	60.98
			AV	34	4	23	7	88.24
M2084	24	Van	Water	34	31	1	2	8.82
		Scoy	Ames	27	9	9	9	66.67
			AV	31	4	18	9	87.10
M2087	24	Van	Water	25	24	1	0	4.00
		Scoy	Ames	33	18	6	9	45.45
			AV	29	2	20	7	93.10
M2088	24	Van	Water	44	41	2	1	6.82
		Scoy	Ames	53	26	10	17	50.94
			AV	49	5	17	27	89.80
M2084	24	Van	Water	51	49	2	0	3.92
		Scoy	Ames	58	17	18	23	53.45
			AV	51	6	25	20	88.24
M2087	24	Van	Water	42	39	3	0	7.14
		Scoy	Ames	37	10	15	12	72.97
			AV	37	4	27	6	89.19
M2088	24	Van	Water	31	28	2	1	9.68
		Scoy	Ames	25	4	4	17	84.00
			AV	35	3	6	26	91.43

Table 32. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	AFB dead	Per cent non-survival
M2083	24	Van Scoy	Water	30	28	2	0	6.67
			Ames	36	11	6	19	69.44
			AV	35	7	6	22	80.00
M2087	24	Van Scoy	Water	43	41	1	1	4.65
			Ames	41	4	8	29	90.24
			AV	44	5	12	27	88.64
M2088	24	Van Scoy	Water	42	38	4	0	9.52
			Ames	48	32	5	11	33.33
			AV	39	4	20	15	89.74
M2087	24	Van Scoy	Water	27	26	0	1	3.70
			Ames	35	23	3	9	34.29
			AV	26	3	10	13	88.46
M2084	24	Van Scoy	Water	49	47	2	0	4.08
			Ames	47	23	8	16	51.06
			AV	48	10	14	24	79.17

Table 33. Raw data of *in vivo* studies of *B. thuringiensis*

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	Non-survivors	Per cent non-survival
M1799	16	Brown	Water	20	19	1	0	5.00
			BT[a]	21	19	2	0	9.52
			Ames	29	5	15	5	82.75
M1799	16	Brown	Water	35	32	3	0	8.10
			BT	48	43	5	0	10.41
			Ames	41	4	14	23	90.24
M1799	24	Brown	Water	61	59	2	0	3.27
			BT	54	54	0	0	0.00
			Ames	64	33	14	17	48.43
M1799	24	Brown	Water	27	25	2	0	7.40
			BT	26	20	6	0	23.07
			Ames	25	3	9	13	88.00
M1799	32	Brown	Water	102	100	1	1	1.96
			BT	84	80	4	0	4.76
			Ames	102	95	4	3	6.86
M1799	32	Brown	Water	30	30	0	0	0.00
			BT	44	41	3	0	6.81
			Ames	26	18	1	7	30.76

[a]*Bacillus thuringiensis*.

Table 33. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	Non-survivors	Per cent non-survival
M1840	16	Van	Water	42	38	3	1	9.52
		Scoy	BT	30	29	1	0	3.33
			Ames	48	3	25	20	93.75
M1840	16	Van	Water	17	16	1	0	5.88
		Scoy	BT	15	14	1	0	6.67
			Ames	31	4	19	8	87.09
M1839	16	Van	Water	22	22	0	0	0.00
		Scoy	BT	16	15	0	1[b]	6.25
			Ames	22	0	16	6	100.00
M1839	16	Van	Water	37	36	1	0	2.70
		Scoy	BT	29	24	4	1[b]	17.24
			Ames	40	2	23	15	95.00
M1840	24	Van	Water	43	40	2	1	6.97
		Scoy	BT	36	35	0	1[b]	2.77
			Ames	37	10	16	11	72.97
M1840	24	Van	Water	36	33	3	0	8.33
		Scoy	BT	29	25	3	1[b]	13.79
			Ames	39	2	23	12	94.59
M1840	32	Van	Water	28	26	2	0	7.14
		Scoy	BT	36	31	5	0	13.88
			Ames	30	21	5	4	30.00

[b] Death caused by AFB.

Table 33. (Continued)

Queen mating number	Age in hours	Line	Treatment	Base count	Living	Missing	Non-survivors	Per cent non-survival
M1840	32	Van Scoy	Water	47	44	3	0	6.38
			BT	37	36	1	0	2.70
			Ames	36	14	9	13	61.11
M1839	32	Van Scoy	Water	31	28	3	0	9.67
			BT	35	30	5	0	14.28
			Ames	30	5	16	9	83.33

Table 34. Number of larvae remaining in combs after various treatments

Queen mating number	Age in hours	Line	Treatment	Days after feeding 0	1	3	5	7	9	11	13
M1799	16	Brown	Water	20	20	20	19	19	19	19	19
			BT[a]	23	21	21	21	21	21	19	19
			Ames	30	29	26	24	24	20	18	14
M1799	16	Brown	Water	47	45	44	42	42	42	42	40
			BT	64	49	45	45	45	44	44	41
			Ames	61	59	55	40	33	32	32	30
M1799	16	Brown	Water	31	30	24	22	22	22	22	22
			BT	21	20	13	12	11	11	11	11
			Ames	28	28	15	9	9	8	8	7
M1799	16	Brown	Water	40	35	35	32	32	32	32	32
			BT	56	48	46	44	43	43	43	43
			Ames	46	41	37	30	29	29	29	27
M1799	24	Brown	Water	61	61	61	59	59	59	59	59
			BT	60	54	54	54	54	54	54	54
			Ames	66	64	63	59	55	51	50	50
M1874	24	Brown	Water	33	28	28	25	25	25	25	24
			BT	42	41	38	35	35	35	34	34
			Ames	34	33	32	28	28	25	22	21
M1799	24	Brown	Water	33	27	27	27	27	27	25	25
			BT	30	26	24	21	21	20	20	20
			Ames	30	25	23	21	20	18	17	16

[a] *Bacillus thuringiensis*.

Table 34. (Continued)

Queen mating number	Age in hours	Line	Treatment	Days after feeding 0	1	3	5	7	9	11	13
M1799	32	Brown	Water	103	103	103	103	102	102	102	102
			BT	85	84	83	83	81	80	80	80
			Ames	102	102	101	101	100	99	99	98
M1874	32	Brown	Water	25	25	24	23	23	23	22	22
			BT	20	20	19	18	18	18	18	18
			Ames	30	30	28	27	27	27	26	25
M1799	32	Brown	Water	32	30	30	30	30	30	30	30
			BT	48	44	42	41	41	41	41	41
			Ames	29	26	26	26	26	26	25	25
M1840	16	Van Scoy	Water	42	42	41	41	41	40	39	39
			BT	36	30	30	30	30	30	29	29
			Ames	52	48	44	34	30	28	24	23
M1840	16	Van Scoy	Water	43	42	41	40	35	34	31	31
			BT	30	26	23	23	23	23	21	21
			Ames	31	29	26	23	21	20	17	16
M1840	16	Van Scoy	Water	45	42	41	39	36	36	36	36
			BT	44	32	32	28	28	28	28	27
			Ames	34	32	30	24	22	20	20	18
M1840	16	Van Scoy	Water	39	39	33	32	31	30	30	30
			BT	35	28	21	20	20	20	20	20
			Ames	37	35	30	23	20	19	19	15

Table 34. (Continued)

Queen mating number	Age in hours	Line	Treatment	Days after feeding 0	1	3	5	7	9	11	13
M1840	24	Van	Water	45	43	43	43	42	42	41	41
		Scoy	BT	38	36	36	36	36	36	36	36
			Ames	37	37	37	31	25	25	22	21
M1840	24	Van	Water	39	36	35	35	33	33	33	33
		Scoy	BT	33	29	28	28	28	27	26	26
			Ames	39	38	36	29	22	21	18	15
M1840	24	Van	Water	33	33	30	23	23	23	23	22
		Scoy	BT	37	30	27	22	22	22	22	22
			Ames	25	23	20	11	11	11	11	10
M1839	24	Van	Water	29	28	17	17	17	17	17	17
		Scoy	BT	29	28	13	10	10	8	8	8
			Ames	34	34	28	24	19	16	15	15
M1840	24	Van	Water	24	24	24	24	23	21	21	21
		Scoy	BT	28	25	25	24	22	22	22	22
			Ames	27	27	27	27	21	14	14	12
M1840	32	Van	Water	29	28	28	27	27	27	27	26
		Scoy	BT	37	36	35	34	32	32	32	31
			Ames	32	30	29	28	25	25	25	25
M1840	32	Van	Water	47	47	46	46	45	44	44	44
		Scoy	BT	39	37	37	37	36	36	36	36
			Ames	39	36	34	33	31	29	28	27

Table 34. (Continued)

Queen mating number	Age in hours	Line	Treatment	Days after feeding							
				0	1	3	5	7	9	11	13
M1840	32	Van Scoy	Water	31	27	23	21	20	20	20	20
			BT	38	35	28	28	26	26	26	26
			Ames	36	35	31	25	22	21	21	19
M1839	32	Van Scoy	Water	31	31	31	31	29	29	28	28
			BT	35	35	32	31	30	30	30	30
			Ames	30	30	30	19	17	16	15	14