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HISTOPATHOLOGICAL EFFECTS OF TWO INSECT CHITIN INHIBITORS IN THE ALIMENTARY CANAL OF CHIRONOMID MIDES (DIPTERA: CHIRONOMIDAE)¹

Frank W. Pelsue²

ABSTRACT. Treatment of selected larvae of Chironomus decorus Johannsen and Tanypus grodhausi Sublette with the insect growth regulators diflubenzuron and Bay SIR-8514 resulted in incomplete ecysis, caused by reduced chitin synthesis, and in a number of histopathological changes in the epithelium of the alimentary canal of both species. The ventriculus of both species was the most severely affected by both compounds, as evidenced by exfoliation of epithelial cells. Destruction of cellular detail was also observed with the cells appearing lacy or reticulate and containing large vacuoles.

INTRODUCTION

The insect growth regulators diflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)-urea) and Bay SIR-8514 (1-(4-trifluoromethoxyphenyl)-3-(2-chlorobenzoyl)-urea) inhibit the enzyme chitin synthetase (Deul et al., 1978). Their activity against a number of insect species is well documented (Mulder and Gijswijt, 1973, Issaaya and Casida, 1974, Arias and Mulla, 1975, Schaefer et al., 1975, Ali and Mulla, 1977, Lacy and Mulla 1978, Mulla and Darwaiez, 1975, and Mulla et al., 1974). In their studies on the insecticidal activity of insect growth regulators, Mulder and Gijswijt (1973) and Arias and Mulla (1975) observed that certain external morphological anomalies were occurring in the test insects at the time of death. At first, since diflubenzuron and Bay SIR-8514 each cause mortality at the larval molt, both compounds were thought to interfere apparently with cuticle deposition, causing the insect to die due to its inability to properly molt. Mulder and Gijswijt (1973), Post et al., (1974), and Ker (1978) have reported a reduction in the thickness of the cuticle in larvae treated with diflubenzuron. Mulder and Gijswijt (1973) also reported that some Pieris brassicae larvae that survive the molt die intramolt due to starvation. They found that these larvae at the time of death lacked fat body. Clark et al., (1978) found that Locusta migratoria had a 70 percent reduction in peritrophic membrane production as compared to that in the check. From the literature, chitin inhibitors possibly appear to be involved not only in preventing the deposition of cuticular chitin, but also in affecting other tissue systems within the insect. Pelsue (1984) reported on morphopathological anomalies in two species of Chironomidae treated with the chitin inhibitors diflubenzuron and Bay SIR-8514. He found that treatment of Chironomus decorus Johannsen with Bay SIR-8514 caused a morphopathological anomaly descriptively called “humpback.” Diflubenzuron did not cause such a pronounced condition. Larvae that died due to diflubenzuron simply failed to complete the molt. Consequently, the expression of morphological malformation was believed to vary from species to species and the chemical test used. In general, the microanatomical literature on the effects of chitin synthesis inhibitors in insects deals with the thickness of the cuticle and the changes in the epidermal cells. Little attention has been paid to other organs or tissue systems. As mentioned earlier, there is some indication that peritrophic membrane synthesis may be affected. Since peritrophic membrane formation may be affected, certain chitin-synthesizing cells in the alimentary canal might be affected by these chemicals. The foregut and hindgut of insects are well known to be lined with cuticle, or intima, continuous with the cuticle covering the body. However, since the intima is highly permeable to water, it does not have the same structure as the integumental cuticle. Nevertheless, as the integumental cuticle is synthesized or forms, so does the intima, both coverings becoming vulnerable to chitin synthesis inhibitors. Because the intima contains chitin, the epithelial cells of the fore and hindgut probably have not only mechanisms that synthesize chitin but also the likelihood of being affected by compounds that block or interfere with chitin synthesis. Another factor that may affect the integrity of the tissues in the alimentary canal is that chitin inhibitors are foreign compounds to the insects and might act as stomach poisons in addition to interfering with chitin synthesis.

From previous investigations, we know that diflubenzuron and Bay SIR-8514 interfere with chitin synthesis and cause morphological anomalies in a number of insect species. The present study was conducted in order to gain an understanding of the effects of chitin inhibitors in producing pathological conditions in the alimentary canal of two species of Chironomidae. This study reports the effects of two insect chitin inhibitors on the gross morphology and microanatomy (histology) of the alimentary canal.

MATERIALS AND METHODS

Two species of chironomid midge larvae were used in this study, Chironomus decorus and Tanypus grodhausi Sublette. Live third and fourth stage larvae used in histopathological examination were obtained from treatment chambers containing various concentrations of diflubenzuron and Bay SIR-8514. Test larvae were fixed either in alcoholic Bouin's fluid or Carnoy's fluid.

¹This study was supported in part by the Southeast Mosquito Abatement District and provided partial fulfillment of the requirements for the Ph.D. degree, Department of Entomology, University of California, Riverside.

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dehydrated in a graded ethanol series and embedded in paraplast.

Tissue sections of four to six microns in thickness were obtained using an American Optical rotary microtome and affixed to microscopic slides for light microscopic examination. Both cross and sagittal sections were obtained. Check larvae were obtained from the untreated test chambers and processed in the same manner as treated larvae. Each treatment for both species and compounds was replicated three times. Tissue sections were stained for both species and compounds with Delafield’s hematoxylin and eosin, Cason’s trichrome, Azure B bromide, and Feulgen reaction for DNA with fast green counterstain.

Slides were examined with a Zeiss® light microscope and photomicrographs were taken with the Zeiss® photomicrographic phase bright-field microscope. Black and white Panatomic-X film was used for documentation of the results.

RESULTS

**Chromonomus decorus**

Figure 1 shows a sagittal section of a larva of this species treated with a concentration of 0.1 ppm Bay SIR-8514 and the characteristic swelling or “humpback” form as reported by Pelsue (1984). The anterior portion of the larva shows a complete disorganization of the new larval tissues within the old larval skin. Internally, there is only an amorphous remnant of the alimentary canal. The head capsule exuviae of the previous larva has an abnormal intrusion of tissue. There appears to be no new definitive head capsule, and the presence of a new larval form inside the previous larval skin is difficult to determine. Epicuticle of the previous larval cuticle remains with the epidermal cells immediately beneath the epicuticle indicating that no new cuticular synthesis has occurred.

Figures 2 through 5 depict the histology in the “hump” area of both treated and check larvae. Figure 2 is a cross section through the “hump” and shows the general tissue and the entire “hump” appears infused with material lacking distinct form, structures being difficult to distinguish. For example, the salivary gland seems to be affected markedly, showing evidence of hypertrophy with an increase in large vacuoles. Its disorganized cytoplasmic matrix contrasts markedly with that of an untreated larva (Fig. 3). Also, in Figure 2, there appears to be only slight formation of new integument. In addition, the esophagus is undefined. As shown in Figure 4, some treated larvae lacking the “hump” anomaly still have abnormal appearing internal organs. For example, the esophagus (Fig. 4) is well defined structurally; however, its intima is abnormally thickened and although the cardial cells that secrete the peritrophic membrane appear unaffected, the caecal cells posterior to them are abnormal (Figs. 4 & 5).

Epithelial cells in the anterior portion of the ventriculus, as depicted in Figure 6, are highly vacuolate and lack definitive form. Nuclei are seen, but most lack defined cell boundaries and some are surrounded by vacuoles. In addition, the cells appear to be shedding into the lumen of the ventriculus. Fat body tissue is also affected and appears extremely reticulate and without definitive form. The posterior ventricular epithelium appears reticulate or lacy and lacks cellular detail, appearing as if it were being pulled apart (Fig. 7). In contrast, Figure 10 shows the well-organized posterior ventricular epithelium in an untreated larva.

Rectal sac tissue in a treated larva appears normal viewed in sagittal section (Fig. 8). However, viewed in cross section, it appears extremely reticulate, having extensive vacuoles and lacking definitive cellular detail (Fig. 9). The absence of vacuoles and presence of well-defined nuclei in a rectal sac of an untreated larva is shown in Figure 11.

Diflubenzuron has an effect on the alimentary canal of *C. decorus* that is different than that of Bay SIR-8514. Since diflubenzuron does not cause the same morphopathological condition (“humpback”) as Bay SIR-8514, we did not observe the same histological disorganization. In diflubenzuron-treated larvae, the esophagus viewed in cross section retains its morphology, as do the cardia, ventriculus, ileum, and rectal sac. However, there are some recognizable histological and cellular differences that can be observed, which occur between the treated and the untreated larva.

The organs with tissues and cells most impaired by diflubenzuron are the ventriculus, caeca, and rectal sac. Figure 12 shows the anterior portion of the ventriculus with irregularly shaped epithelial cells, many seen shedding into the lumen. The untreated larva shows no such cellular structure (Figs. 14 & 15). Cellular integrity is maintained in mid and posterior ventricular epithelium of untreated larvae (Fig. 18). Cells of the rectal sac epithelium have a somewhat reticulate, highly vacuolate appearance similar to those of the epithelium of the ventriculus. Untreated larvae have rectal sacs possessing well-defined epithelial cells containing homogeneous cytoplasm and few small vacuoles.

**Tanyurus groehausi**

Bay SIR-8514 does not cause the same disruption in the tissues of the alimentary canal of this species that it causes in *C. decorus*. In *T. groehausi*, from the head capsule back to the cardia, there is a significant disorganization of the tissues. Figure 21 shows the tissue disarray anterior to the cardia. Figures 22 and 23 depict similar areas of the cardia in treated and untreated larvae, respectively. The caecal pouches are still distinguishable, but there appears to be shedding of the ventricular epithelial cells into the gut lumen. In treated larvae, the caecal epithelial cells have vacuoles near their apex, and they lack the eosinophilic granule (Fig. 24) as found in similar cells of untreated larvae (Fig. 25). Posterior to the caeca, the cells of the ventriculus appear to be proliferating and shedding into the gut lumen (Fig. 26), a condition not evident in untreated larvae (Fig. 27).

In mid ventriculus, the epithelial cells appear not to be shedding; however, they are becoming more columnar or elongated (Fig. 28), some quite different from those in untreated larvae (Fig. 29). Here, too, the cells appear highly vaculate, containing diffuse granular bodies (Fig. 30). Viewed in cross section, the posterior ventriculus anterior to the pyloric valve has epithelial cells that are more cuboidal and which contain many vacuoles and diffuse granules. Posterior to the pyloric valve, the epithelial cells, as shown in Figure 31, appear reticulate and lack definitive form. The lumen seems to lack the heavy coagulated intima (Fig. 32) compared with that in untreated larvae (Fig. 33). The rectal sac epithelium appears flattened, having occasional cells protruding into the gut lumen (Fig. 35). In contrast, rectal sac epithelium in untreated larvae has the appearance of papillae-like folds with cells having large apical nuclei (Fig. 34).

The effects of diflubenzuron on the alimentary canal of *T. groehausi* are similar to that found in *C. decorus*.
Figure 1. Sagittal section of *C. decorus* showing “hump” (thick arrow) and disorganization of midgut (VE = ventricular epithelium, thin arrow) and other tissue in anterior portion of larvae. Treatment with Bay SIR-8514.

Figure 2. Cross section of *C. decorus* illustrating general cellular disorganization. Long arrow points out empty head capsule of previous larval stadium. Short arrow SG (salivary gland) points out the salivary gland. Treatment with Bay SIR-8514.

Figure 3. Cross section of anterior portion of untreated (check) of *C. decorus* depicting normal organization of salivary gland.

Figure 4. Sagittal section showing thickened intima of the esophagus (arrow), cardiac valve (CV), and the cardia (C) of larval *C. decorus* that died due to Bay SIR-8514 treatment. Larva lacks morphopathological anomaly.

Figure 5. Cross section of cardia of *C. decorus* treated with Bay SIR-8514.

Figure 6. Anterior ventriculus of *C. decorus* illustrating epithelial cell shedding and the large vacuoles present within the cells. Larva treated with Bay SIR-8514.
Figure 7. Posterior ventricular epithelium of *C. decorus* treated with Bay SIR-8514 illustrating the lack of cellular detail and large reticulate cells containing vacuoles.

Figure 8. Rectal sac of *C. decorus* in sagittal section showing a rather normal cellular configuration after treatment with Bay SIR-8514. Hematoxylin and Eosin (H and E).

Figure 9. Rectal sac of *C. decorus* in cross section illustrating large vacuoles and lack of cellular detail after treatment with Bay SIR-8514.

Figure 10. Untreated *C. decorus* showing the normal posterior ventricular epithelium. Azure B.

Figure 11. Untreated *C. decorus* rectal sac in cross section. Azure B.

Figure 12. Anterior ventriculus of *C. decorus* showing cellular shedding and general deformity after treatment with diflubenzuron. Epithelial cells lack large vacuoles. Azure B.
Figure 13. Untreated *C. decorus* showing normal anterior ventriculus. Azure B.

Figure 14. Diflubenzuron treated *C. decorus* illustrating midventriculus. Cells lack cellular detail and contain some vacuoles. Azure B.

Figure 15. Posterior midgut (ventriculus) of *C. decorus* treated with diflubenzuron showing somewhat more numerous vacuoles but not appearing reticulate as in Bay SIR-8514 treated larvae.

Figure 16. Untreated *C. decorus* midventriculus. Azure B.

Figure 17. Cross section of untreated *C. decorus* illustrating normal posterior ventriculus. Azure B.

Figure 18. Caeca of *C. decorus* treated with diflubenzuron. Compacting of the pouches is not normal. Azure B.

Figure 19. Rectal sac of *C. decorus* showing the lack of deep staining and presence of large vacuoles. Larva treated with diflubenzuron. Azure B.

Figure 20. Cross section of rectal sac of untreated *C. decorus* larva. Azure B.
Figure 21. Cardial cells of *T. grodhausi* (arrow) treated with Bay SIR-8514 illustrating the hypertrophy of the cells and the large intracellular spaces. Feulgen, Fast Green.

Figure 22. Tentacle cells of *T. grodhausi* (arrow) treated with Bay SIR-8514 illustrating a deformity and lack of definitive endoplasmic reticulum. Feulgen, Fast Green.

Figure 22a. Tentacle cell. Untreated larva. H and E.
Figure 23. Cross section of the cardia of untreated *T. grodhausi*. H and E.

Figure 24. Caeca of *T. grodhausi* treated with Bay SIR-8514 illustrating vacuoles in the apical cytoplasm of the epithelium and the lack of a large vacuole containing a large eosinophilic granule as seen in Fig. 3-25 (arrow). Azure B.

Figure 25. Untreated *T. grodhausi* showing normal configuration of caeca and cells containing eosinophilic granule (arrow). H and E.

Figure 26. Cross section of ventriculus of *T. grodhausi* illustrating the effects of Bay SIR-8514. Epithelial cells appear reticulate and lack definitive cell structure. Azure B.

Figure 27. Cross section of anterior ventriculus of untreated *T. grodhausi* showing vacuole containing eosinophilic granule (arrow). H and E.

Figure 28. Midventriculus of *T. grodhausi* treated with Bay SIR-8514. Azure B.
Figure 29. Cross section of untreated *T. grodhausi* illustrating midventriculus with its cuticular intima (large arrow) and the prominent eosinophilic granule in large vacuole. Azure B.

Figure 30. Photomicrograph of diffuse granules present in ventriculus of *T. grodhausi* treated with Bay SIR-8514. Azure B.

Figure 31. Posterior ventriculus (arrow) of *T. grodhausi* treated with Bay SIR-8514. Azure B.

Figure 32. Cross section of *T. grodhausi* after treatment showing the ileum (large arrow) and lack of cuticular intima. Azure B.

Figure 33. Untreated *T. grodhausi* showing ileum (large arrow) and malpighian tubules (short arrow). H and E.

Figure 34. Rectal sac of untreated *T. grodhausi* depicting the prominent papillae (arrow). Azure B.

Figure 35. Rectal sac of *T. grodhausi* treated with Bay SIR-8514 showing the lack of or reduction in the rectal papillae (large arrow) and malpighian tubules (short arrow). Azure B.
Figure 36. Sagittal section of *T. grodhausi* depicting anterior portion of larva responding to treatment with diflubenzuron and showing somewhat normal organization. Arrows indicate esophagus (E), cardia (C), and tentacle cells (T). H and E.

Figure 37. Sagittal section of *T. grodhausi* showing atrophied caeca appearing somewhat reticulate after treatment with diflubenzuron. Azure B.

Figure 38. Posterior ventriculus after diflubenzuron treatment showing some reticulate cells. *T. grodhausi*. H and E.

Figure 39. Ileum of *T. grodhausi* after diflubenzuron treatment showing the reduction in musculature and lack of cuticular intima. Feulgen, Fast Green.

Figure 40. Rectal sac of *T. grodhausi* treated with diflubenzuron showing reduction in papillae as compared to untreated larvae. Azure B.
No gross anterior morphological distortions (as occurred from the effects of Bay SIR-8514) appear in the larvae or their internal organs, including the esophagus, cardia, and tentacle cells. All these structures are readily recognized when viewed in sagittal and cross section. However, some histological and cytological abnormalities do occur. Tentacle epithelial cell cytoplasm is reticulate (Fig. 36), as is that in the caeca, making the pouches appear atrophied (Fig. 37).

In T. grodhausi, the gut organs behind the caeca also seem affected histologically and cytologically by diflubenzuron. Ventricular epithelial cells appear reticulate and lack the characteristic granule usually present within a large vacuole. The posterior ventriculus shows the same reticulate epithelial cell cytoplasm and general lack of cellular detail as other portions of the ventriculus (Fig. 38). The muscular sheath of the ileum appears thinner than that in the check and lacks the convoluted intima, which is usually present (Fig. 39). Rectal and caecal epithelial cells appear similar to those in the Bay SIR-8514 treated larvae in that they lack definitive form and the peritrophic membrane or convoluted intima is absent.

**DISCUSSION**

The evidence presented here indicates that both diflubenzuron and Bay SIR-8514 cause pathological conditions in the epithelial canal of C. decorus and T. grodhausi. In the former species, Bay SIR-8514 tends to cause dramatic disorganization in the area of the "hump." The tissues in the area of the "hump" lack the essential form that is found in the alimentary canals of the untreated larvae. The epithelial cells of the anterior ventriculus that are distinguishable in treated larvae show considerable irregularities compared to those of untreated larvae, including a lack of typical cell morphology, some cellular shedding, and the occurrence of large apical vacuoles. In T. grodhausi, Bay SIR-8514 causes similar cellular deformities in the anterior gut, but most cellular zones remain identifiable, with well-defined organs, i.e., the esophagus, cardia, tentacles, and caeca. However, some anterior gut epithelial tissues and cells appear abnormal. The esophageal epithelium is abnormally thickened, and the cardial cells appear basophilic and diffuse instead of compact. The tentacle epithelial cells lack cytoplasm and appear ill-defined. The caecal pouches are atrophied and their epithelial cells appear somewhat elongated, lacking the vacuole and secretory granule, which is present in the cells of untreated larvae. Other gut epithelial cells appear abnormal, particularly the posterior ventricular cells, which lack definitive form and have a reticulate, diffuse cytoplasm.

The effect of diflubenzuron in producing pathological conditions is very similar in both species studied. Since diflubenzuron does not cause the "humpback" condition in either species, the organization of the alimentary canal is not radically disrupted in larvae that die while molting. However, there is damage to certain cells of the alimentary canal in both C. decorus and T. grodhausi. There are similarities between the type of abnormalities caused by both compounds in each species. In both species, cells that are dramatically affected appear reticulate and lack definitive form. Certain portions of the affected tissues shed into the gut lumen. Both materials cause the greatest pathological conditions in the ventricular epithelial cells. This is rather surprising since the structure of the ventriculus is not usually altered during ecdysis. Cells of the cardia do not appear to be affected markedly in either species but seem to be somewhat diffuse in T. grodhausi treated with diflubenzuron. The effects of both materials on the hindgut cause a reduced and misshaped rectal sac in T. grodhausi and somewhat distorted epithelial cells in C. decorus, with a complete absence of chitinous intima in both species.

Since the ventriculus is the organ most severely affected and normally lacks chitin, we conclude that both diflubenzuron and Bay SIR-8514 at lethal concentrations cause an effect other than the inhibition of chitin synthetase. We hypothesize that both compounds also act as a mild stomach poison and have some effect on the ability of the larvae to feed properly. Pelsue (1984) has shown that both compounds affect feeding activity. The larvae of C. decorus, after being treated with either compound and surviving a molt where over 50 percent of the treated population are killed, attempt to feed but do not ingest any food material and ultimately die of starvation before the next molt. The results of this investigation illustrate that diflubenzuron and Bay SIR-8514 cause histopathological conditions that can contribute to mortality besides reducing the amount of chitin synthesized in the cuticle. Both materials can cause shedding of ventricular epithelial cells and hypertrrophy of the salivary glands with cells containing large cytoplasmic vacuoles. Epithelial cells of the posterior ventriculus appear reticulate and lack definitive form, thus, contributing to a general breakdown and dysfunction of the digestive apparatus.

**Acknowledgements**

Appreciation is extended to the Board of Trustees of the Southeast Mosquito Abatement District for their generous support during this study. In addition, I thank Dr. Mir S. Mulla, Department of Entomology, University of California, Riverside, for his support and direction during this research.

**REFERENCES CITED**


RECOGNITION AND ISOLATION OF *LAGENIDIUM GIGANTEUM* COUCH

P. T. Brey and G. Remaudiere

ABSTRACT. A protocol is presented for the recognition and isolation of *Lagenidium giganteum*, a facultative fungal pathogen of mosquito larvae. Prospection, recognition, and isolation techniques are explained in detail with the aid of photo micrographs. This paper a guide to locate and put into culture new isolates of this aquatic fungus.

INTRODUCTION

*Lagenidium giganteum* Couch (Oomycetes) is a facultative fungal pathogen of mosquito larvae. Originally described by Couch (1935), this aquatic fungus has only been seriously considered as a potential biocontrol agent since the early 1970's. Continual progress made on its basic pathogenic and optimistic preliminary field trials have presently positioned this oomycetous fungus as one of the promising fungal agents for the biological control of mosquito larvae (W.H.O., 1984).

In establishing biological control agents, it is indispensable to have as many strains as possible so that the most virulent and best adapted to a given vector species in a given environment may be selected. Presently, there are only four isolates of *L. giganteum* in experimental use; all four originating from the temperate zone of North America (TABLE 1). Other isolates of *L. giganteum* have been discovered throughout the world: In India, in mosquito larvae related by Couch and Romney (1973); in the United Kingdom; Uganda and the South Orkneys Islands in Antarctica; isolated from termite wing baits by Willoughby (1969). However, these isolates were not maintained in culture, therefore, making it impossible to confirm their pathogenic activity. It is most probable that the restricted natural distribution of mosquito infecting *L. giganteum* isolates is simply due to the lack of active and persistent prospection throughout other regions of the world.

Whether the existing North American *L. giganteum* isolates will be fully adapted to tropical mosquito breeding sites is unknown, therefore, emphasizing the importance of obtaining *L. giganteum* isolates indigenous to torrid zones.

The purpose of this paper is to call attention to the ever present need for new isolates of *L. giganteum* and to explain with the aid of photographs how to look for suspected *L. giganteum* isolates, how to recognize and isolate them in pure culture.

PROSPECTION PROCEDURE

The naturally occurring North American isolates of *L. giganteum* were found, for the most part, accidentally during routine larval collections. In such cases, the person collecting the mosquito larvae encountered a natural epizootic of *L. giganteum*, manifested by high larval mortality, i.e., many larval cadavers floating on water surface. Due to fluctuations of interdependent ecological factors, the epizootic is a sporadic event. Hence, low level infection maintaining the pathogenesis within a biotope can easily go undetected if not specifically looked for.

Prospection Site Selection

*Lagenidium giganteum* prospection should be conducted in permanent or semi-permanent larval breeding sites where complete drying is occasional. The water of the breeding site should preferentially be free from organic pollution and salinity. These hydrochemical conditions have been found to have adverse effects on the North American isolates of *L. giganteum* (Merriam and Axtell, 1982; Jaronski and Axtell, 1982). Once the prospection sites have been selected, two methods can be used to detect *L. giganteum* and other fungal pathogens therein.

Method 1: Larvae collected from the breeding site should be separated according to species, or at least genera, and placed in rearing pans or other containers half filled with water from the collection site. The water used for rearing contains sufficient food for the holding period so feeding is not required. Larvae should be held at 25 ± 3°C for 48 hours. During this period any dead or moribund larvae should be examined microscopically (10-40X). A high number of field collected larvae will favor the probability of finding infected larvae.

Method 2: Another method to detect *L. giganteum* or other fungal pathogens in a given breeding site is to introduce sentinel larvae as previously used by Jaronski and Axtell (1984). This method requires healthy laboratory reared Culicine or Anopheline larvae or those taken from another breeding site proven negative for *L. giganteum* and sentinel containers, i.e., floatable screened cups, which allow external water to flow through the cup (Fig. 1). Sentinel larvae (3-5 instar) are introduced into the suspected site in the screened sentinel containers (50 larvae/container/4m² of surface water). The containers are allowed to float freely in the water for 48 hours. Any dead or moribund larvae should be examined microscopically (10-40X).

Recognition of *L. giganteum* infecting mosquito larvae

*Lagenidium giganteum* exhibits an asexual and sexual phase of reproduction, both of which are ongoing in the same larval host (Fig. 2). Both phases are initiated by a laterally biflagellate motile zoospore. *L. giganteum* was described by Couch (1935) and will be discussed in detail in this paper.

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1 Unité de Lutte Biologique contre les Insectes I, Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris Cedex 15, FRANCE.
TABLE 1. *Lagenidium giganteum* isolates available for experimental use.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolation Information</th>
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<tbody>
<tr>
<td>Louisiana (LgLA)</td>
<td>Isolated by F. E. Glenn and H. C. Chapman from <em>Culex territans</em> in a Black Gum swamp near Moss Bluff, LA in 1975. This isolate corresponds to N° 48336 ATCC.</td>
</tr>
<tr>
<td>North Carolina (LgNC)</td>
<td>Isolated by A. L. Knight from mosquito larvae in Chapel Hill, NC in 1979. This isolate corresponds to N° 48337 ATCC.</td>
</tr>
<tr>
<td>California (LgCA)</td>
<td>Isolated by R. Washino and colleagues from mosquito larvae near Colusa, CA in 1979. This isolate is thought to be the descendant of another North Carolina isolate provided by C. J. Umphlett and E. M. McCray, which was originally introduced into this locale in 1972. This isolate corresponds to N° 52675 ATCC.</td>
</tr>
</tbody>
</table>

1 American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A.

zoospores are usually ovoid, measuring 8.9–9.0 μm width × 9.1–9.9 μm length. The swimming of the zoospores is often erratic, i.e., zigzagging with abrupt stops and starts. When the zoospore makes contact with the larval host, it adheres to the cuticle and encysts. The encysted zoospore puts forth a germ tube, which penetrates the larval cuticle (Fig. 3).

Once the cuticular barrier is crossed, the germ tube gives rise to a nonsegmented branching vegetative fungal filament or hypha (10–20 μm dia.) leaving behind the empty zoospore cyst on the cuticle surface (Fig. 4). Following germ tube penetration and initial hyphal colonization, the invading hyphae provoke larval melanization reactions. The reactions are manifested by darkening of the cuticle around the penetration points and also on the hyphal surface, facilitating the recognition of fungal infection. After the hyphae have become numerous and interwoven, cross walls or septa are laid down dividing the hyphae into segments (Fig. 5). With the hyphae compartmentalized, each segment swells to 20–40 μm diameter while remaining constricted at the septa. This often gives the hyphae a bead-like appearance (Figs. 6 & 7). The hyphal segments will give rise to either sporangia (asexual phase) or gametangia (sexual phase). When asexual reproduction takes place, the sporangia form exit tubes, which make their way through cuticle into the external aquatic environment. The contents of the sporangia are evacuated through the exit tube forming a terminal vesicle approximately 30 μm diameter at the apex. The sporangial contents in the vesicle rapidly cleave into 8–30 zoospores (Fig. 8). The frantic movement of the zoospores ruptures the vesicle releasing the zoospores into the external environment leaving behind the empty exit tubes (Fig. 9). When sexual reproduction occurs, the antheridium "♂" makes contact with the oogonium "♀" via a fertilization tube and injects its contents into the oogonium. The mixture of the "♂" and "♀" gametes give rise to a zygote (Fig. 8), which will mature into a oospore after approximately seven days (Fig. 10).

The most salient features used to recognize *L. giganteum* in mosquito larvae are the following:

- Melanization spots in head capsule and over cuticle surface, melanization tracks mostly in head capsule.
- Segmented hyphae (20–40 μm diameter) constricted at septa.
- Biflagellate motile zoospores (8.9–9.0 μm diameter × 9.1–9.9 μm length) produced within vesicles (ca 30 μm diameter) at tip of an exit tube originating from sporangium.
- Zygotes (15–20 μm diameter) remaining within an oogonium, maturing into fully formed oospores.

**ISOLATION**

If dead or moribund larvae are found to be infected with what appears to be *L. giganteum* or another fungal pathogen, attempts to isolate the fungus should be made. See Figure 11 for isolation technique diagram.

Firstly, it is necessary to confirm that the fungus "infecting" the larvae is truly a pathogen and not a saprophyte. The infected larva or larvae should be added to a recipient containing 50 healthy larvae. If the fungus is a true pathogen, the infection should propagate throughout the larval population providing sufficient inoculum for isolation. Twenty-four to forty-eight hours after exposure to the original propagation inoculum, all additional dead or moribund larvae should be examined microscopically. If hyphae are present in the body cavity, isolation may proceed.

Infected larvae are transferred individually into a petri dish containing 70 percent ethanol for 30 seconds to surface sterilize the larval body. Submersion in a 10 percent dilution of hypochlorite bleach (1 mM) can also be used for surface sterilization. Each larva is transferred twice into tubes containing sterile distilled water to remove excess alcohol. The infected cadaver is then aseptically inoculated onto PYG agar medium and incubated for 24 hours at ambient temperature to allow the fungus to grow onto the medium. To observe the fungus on the agar medium, examination of the tube under a stereomicroscope (×25) is necessary. At least 25 tubes should be initially inoculated, each one with an infected larva, as only a few will have hyphae growing out from them and others will be contaminated. When hyphae are found on the agar surface, a small portion from the periphery of the culture should be transferred to a fresh tube of medium for pure fungal culture. The reason hyphae should be taken from the periphery of the
Figure 1. Diagrammatic representation of the fabrication of sentinel containers.
Figure 2. Life cycle of *L. giganteum*, a modified version of McCray, et al. (1973).
Figure 3. Encysted zoospore (EZ) of *L. giganteum* (LgCA) giving rise to penetrating germ tube (GT) in contact with the cuticle (C) of a 3rd instar *Aedes aegypti* larva. Note empty zoospore cysts (ZC) remaining on the cuticle surface.

Figure 4. Nonseptate penetrating hyphae (H) of *L. giganteum* (LgCA) within the body cavity – hemocoel (Hc) of a 3rd instar *Aedes aegypti* larva. Note empty zoospore cysts (ZC) and melanized areas (M) of the cuticle (C) where the germ tubes have penetrated.

Figure 5. Hyphae (H) of *L. giganteum* (LgCA) invading an anal gill (AG) of a 3rd instar *Aedes aegypti* larva. Note the beginning of compartmentalization of hyphal segments by septa (S).

Figure 6. Completely segmented hyphae (H) of *L. giganteum* (LgCA) invading the hemocoel (Hc) of an *Aedes aegypti* 3rd instar larva. Note bead-like appearance due to swelling of individual segments while remaining constricted at the septa (S).

Figure 7. Segmented hyphae (H) of *L. giganteum* (LgCA) in the respiratory siphon (RS) of a 3rd instar *Aedes aegypti* larva.

Figure 8. Segmented hyphae of *L. giganteum* (LgCA) in *Aedes aegypti* 3rd instar larva tranforming into sporangia (Sp) (asexual phase) and gametangia (G, "♂"; "♀") (sexual phase). Exit tubes (ET) originating from sporangia, progressing into external aquatic environment. Cleavage of zoospores (Z) within terminal vesicle (V). Note zygotes (Zg) within the oogonia (O).

Figure 9. Empty exit tubes (ET) outside the larval body after zoospore released. Cuticle (C).

Figure 10. Mature zygotes – Oospores (OS) within oogonia (O). Note the thick oospore wall (OW), the fine-grained cytoplasm (Cp), and the central lipid globule (LG).
Figure 11. A diagrammatic representation of a technique to isolate facultative fungal pathogens from mosquito larvae. This procedure must be performed near a Bunsen burner or, preferably, under a transfer hood.
culture is to reduce the chances of bacterial and saprophytic fungal contamination. Growth of L. giganteum on PYG agar supplemented with antibiotics is slow, 1.0 cm after seven days. The L. giganteum mycelium growing out from the larval cadaver is composed of hyphal filaments 8-25 µm diameter. These filaments are irregularly branched with numerous septations, hyphae become contracted at the segments taking on a head-like appearance much like that observed in vivo (Figs. 6 & 7). Fungal growth is of an open type remaining close to the agar surface with no aerial structures. The L. giganteum culture on this medium is white to cream colored. Fast growing (>1 cm/day), cottony white or dark colored fungal colonies are most likely saprophytic contaminants and should be discarded. Pure fungal cultures should be sent to one of the laboratories actively working with the fungus for identification2. It is also suggested that persons having found what appears to be L. giganteum, but unable to isolate the fungus, should contact the above mentioned laboratories, and if possible forward freshly infected larvae in a glycerol/water solution (50/50 V/V) for subsequent isolation and identification.

Acknowledgements

This investigation received financial support from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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2PYG agar medium can be made by adding peptone 1.25 g, yeast extract 1.25 g, glucose, 3.0 g and 20.0 g, agar into 900 ml of distilled water. The mixture should be stirred thoroughly. Once the peptone, yeast extract, and glucose are completely dissolved, the broth mixture should be autoclaved at 114°C for 20 minutes. Following autoclaving, the sterilized broth medium is allowed to cool to touch (50°C). At this time antibiotics, d-Penicillin V 0.5 g and Streptomycin Sulfate 0.5 g should be added to 100 ml of distilled water (50°C) and stirred until the antibiotics are in solution. The antibiotic solution can then be added aseptically, via syringe adapted millipore filters, into the cooling broth medium to bring the final volume up to a liter. With antibiotics added, the PYG broth should be swirled to obtain a homogenous mixture. The medium can then be distributed into sterile tubes plugged with sterile cotton and allowed to solidify.

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THE EFFECTS OF GAMMA RADIATION ON SPORE VIABILITY AND MOSQUITO LARVICIDAL ACTIVITY OF BACILLUS SPHAERICUS AND BACILLUS THURINGIENSIS VAR. ISRAELIENSIS

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ABSTRACT. Gamma irradiation of 50-1750 Kr progressively lowered spore viability in Bacillus sphaericus (2362) and Bacillus thuringiensis (H-14) spore powders. A statistically significant loss of mosquito larvicidal activity was observed in B. thuringiensis (H-14) preparations exposed to radiation over 1000 Kr. An insignificant loss of activity was observed in B. sphaericus preparations at the highest dosages of radiation.

INTRODUCTION

The literature is replete with documentation of the efficacy of Bacillus thuringiensis var. israelensis serotype H-14 for the control of black flies and mosquitoes (Gaugler and Pinney 1982 and Lacey 1985) and Bacillus sphaericus for the control of several species of mosquitoes (Davidson 1984). In certain mosquito control situations, most notably when treating water that is used for human consumption, it may become advisable or even mandatory to use sterilized bacterial preparations in which the mosquito toxins are left intact. The sterilizing effect of radiation on entomopathogens is well documented (Burges et al., 1975, Burke et al., 1983, Engler et al., 1980, Ignoffo et al., 1977, and Krieg et al., 1981). Exposure of B. thuringiensis (H-14) and B. sphaericus to sufficient quantities of solar radiation may result not only in spore inactivation but also in loss of insecticidal activity (Ignoffo et al., 1981 and Mulligan et al., 1980). Irradiation of B. sphaericus and B. thuringiensis (H-14) with ultraviolet rays can, however, sharply reduce spore viability without a noticeable loss in larvicidal activity (Burke et al., 1983 and Krieg et al., 1980). Similar findings were reported by Burges et al., (1975) for B. thuringiensis (H-5) after gamma and UV irradiation. UV and gamma irradiated preparations of B. thuringiensis (H-14) were highly effective under field conditions for control of mosquito larvae by Engler et al., (1980) and Schnetter et al., (1981, 1983), respectively. No published reports, however, are available on the effects of gamma radiation on spore viability and mosquito larvicidal activity in mosquito active isolates of B. sphaericus. It was the objective of our study to quantify the effects of gamma radiation on dry, unrefined preparations of B. sphaericus and B. thuringiensis (H-14) with respect to mosquito larvicidal activity and spore viability.

MATERIALS AND METHODS

The bacterial preparations used in our research were: spray dried primary powder of B. sphaericus, isolate 2362 provided by Dr. H. T. Dulmage, USDA, ARS, Brownsville, TX, U.S.A. and air dried B. thuringiensis (H-14) international standard IPS-78, provided by Dr. H. de Barjac, Pasteur Institute, Paris, France. Several 20 ml liquid scintillation glass vials, each containing one gram of either B. sphaericus or B. thuringiensis (H-14) powder were exposed to variable amounts of gamma radiation (50-1750 Kilorontgens [Kr]) from a Cobalt-60 source in a Gammacell R 220 irradiator at a dose rate of 2000 roentgens/min. The entire process was replicated two weeks later for both bacteria for all levels of irradiation.

The two lots of each bacterium were bioassayed against mosquito larvae using standard bioassay procedures (Lacey 1984 and McLaughlin et al., 1984). Samples of nonirradiated and irradiated B. thuringiensis (H-14) powder were bioassayed against early 4th instar Aedes aegypti. Twenty larvae per cup in 100 ml of well water, three cups per level of radiation, were exposed to 0.4 mg/liter (ppm) of the various aliquots of bacterial preparation without the addition of food as recommended by McLaughlin et al., (1984). Three cups of 20 untreated larvae were used as controls. The larvae were held at 27°C and mortality was determined after 24 hours. Four replicate tests were conducted on separate days, two for each irradiated lot. The protocol for bioassay of nonirradiated and irradiated B. sphaericus differed from that of the B. thuringiensis (H-14) bioassay in that 48 hour old 2nd instars of Culex quinquefasciatus were exposed to 0.01 mg/liter (ppm) of spore powder for 48 hours with the addition of 50 mg of debittered Brewer's yeast per cup as larval food. All other conditions were identical.

The effects of the various dosages of gamma radiation on the viability of B. sphaericus and B. thuringiensis (H-14) spores were determined by simple tests for germination and colony formation. After pasteurization in distilled water (80°C for 12 min.), spore suspensions of B. thuringiensis (H-14) and B. sphaericus were serially diluted and plated on tryptose blood agar base and nutrient agar with yeast extract, respectively. After incubating the plates at 35°C for 48 hours, bacterial colonies were counted. Data were analyzed for correlation between radiation dose and reduction of viable spores and between radiation dose and reduction of larvicidal activity using the SAS general linear models procedure.

RESULTS

Figure 1 depicts both larvicidal activity and spore viability of B. sphaericus after aliquots of spray dried

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Figure 1. Effect of gamma irradiation on spore viability and mosquito larvicidal activity against *Culex quinquefasciatus* of a spray dried preparation of *Bacillus sphaericus* (2362). Bioassays were conducted with 0.01 mg/l of the 2362 preparation.

Figure 2. Effect of gamma irradiation of spore viability and mosquito larvicidal activity against *Aedes aegypti* of a spray dried preparation (IPS-78) of *Bacillus thuringiensis* (H-14). Bioassays were conducted with 0.4 mg/l of the IPS-78 preparation.
primary powder were irradiated with 50-1750 Kr of gamma radiation. There was a strong positive correlation between dosage of radiation and reduction in viable B. sphaericus spore count ($R^2 = 0.98$, slope = $1.63 \times 10^{-3}$). Although a slight reduction in larvicidal activity at the higher radiation doses is apparent ($R^2 = 0.37$, slope = $-0.01$), the loss of activity was not significant ($P < 0.05$).

Figure 2 presents data on the effect of gamma radiation (50-1750 Kr) on spore viability and larvicidal activity in B. thuringiensis (H-14). A sharp and steady reduction of spore viability was observed with the IPS-78 powders that had been irradiated with increasing doses of gamma radiation ($R^2 = 0.98$, slope = $3.77 \times 10^{-4}$). A statistically significant reduction in larvicidal activity was also observed ($R^2 = 0.71; P < 0.01$) at exposures above 1000 Kr.

Special attention should be given to the two scales used to present spore viability and larvicidal activity. The use of logarithmic and linear scales on the same graph could be misleading if one fails to note the differences in the scales with respect to the comparable degree of reduction in the two variables. In Figure 1 for example, at 1500 Kr there is a 99.999% reduction in viable spores, yet only a 17% reduction in larvicidal activity.

**DISCUSSION**

The damage caused by gamma radiation in bacteria is due principally to random ionizing of any of several cell constituents. Ultraviolet radiation, on the other hand, affects bacteria through absorption of energy by the nucleic acids (Bridges, 1976). The fairly high levels of larvicidal activity remaining in the preparations of B. sphaericus and B. thuringiensis after irradiation at 1750 Kr indicate, as did the work of Burges et al. (1975) and Burke et al. (1983) that viable spores of either bacterium are not essential for insecticidal activity. In previous research on purified spores and parasporal crystalline inclusions of B. thuringiensis that were exposed to gamma and UV radiation (Burges et al., 1975) and on purified spores of B. sphaericus exposed to UV radiation (Burke et al., 1983) no loss of insecticidal activity was reported in samples in which most of the spores had been killed. Unrefined bacterial preparations were used for our study in order to better approximate operational mosquito control conditions. The loss of larvicidal activity that was observed for the two preparations exposed to the higher dosages of radiation ($\geq 1000$ Kr) in our research may be due in part to the unpurified nature of the powders and the interactive effects of ionizing radiation on the bacterial toxins, nutrients and other substances in the fermentation residues. Similarly, irradiation of whole cultures of B. thuringiensis with 2000 Kr of gamma rays by Panbangred et al. (1979) resulted in a five-fold decrease in larvicidal activity against Ae. aegypti. As in our study, no decrease in activity was noted by them in whole cultures exposed to dosages equal to or less than 1000 Kr.

It is interesting to note that larvicidal activity in aqueous suspensions of B. sphaericus spore powder was lost when they were exposed to sunlight for 5-6 hours (Mulligan et al., 1980). Burke et al. (1983) speculate that the above mentioned loss of insecticidal activity may have been due to germination of the B. sphaericus spores under the warm, organically enriched conditions in the exposure medium. They cited work by Myers et al. (1979) in which it was demonstrated that vegetative cells of B. sphaericus are considerably less insecticidal than spores.

One advantage of using sterilized formulations of Bacillus insect pathogens in certain aquatic habitats would be the reduction of viable cell count, which may be required in public water systems in some communities. Another advantage could be a form of copyright protection for genetically engineered bacterial larvicides. A possible disadvantage with B. sphaericus is the obvious effect that spore deactivation would have on the organism's ability to recycle; however, in some circumstances this might be advantageous. Based on our results, it is feasible to drastically reduce the viable spore count of primary powders of B. thuringiensis and B. sphaericus without an acceptable loss of larvicidal activity.

**Acknowledgements**

We wish to thank Drs. H. T. Dulmage and H. de Barjac for providing the bacterial preparations. Technical assistance was provided by Ms. Loretta Callan and Ms. Genie Avery. We are grateful for the review of our manuscript by Dr. William F. Burke, Jr., Arizona State University, and Donald L. Bailey, USDA, ARS. This research was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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ABSTRACT. Bacillus sphaericus Neide spores were held in citrate, phosphate, and carbonate buffers ranging from pH 3 to 10.8 at 4° and 21°C for up to 308 days to assess the effect of pH and temperature on larvicidal activity and spore viability. Spore counts of the suspensions and larvicidal activity against 2nd instar Culex quinquefasciatus Say exposed to the suspensions remained relatively high for as long as three months for spores held at 4°C in pH 3 to 10. Those held at 4° and 21°C in pH 10.8 lost larvicidal activity within the first week but spore counts remained high. After 308 days, larvicidal activity remained relatively high for spores stored at 4°C in buffers ranging from pH 3 to 8 and spore counts remained high in buffers ranging from pH 5 to 10.8. Loss of larvicidal activity and decline in spore viability in the buffers held at 21°C was significantly accelerated over that of the group held at 4°C. Only negligible larvicidal activity was observed for spores in the 21°C group when tests were terminated after 240 days. The best storage regime for preservation of both spore viability and toxicity was 4°C in neutral buffer.

INTRODUCTION
The mosquito larvicidal toxin and spores of Bacillus sphaericus Neide are capable of persisting for a considerable length of time under a variety of conditions (Hertlein et al., 1979, Mulligan et al., 1980, Silapanuntakul et al., 1983, Davidson et al., 1984, Mull, et al., 1984, and Vankova, 1984). Under storage conditions, especially when the spore preparation is dry and/or refrigerated, larvicidal activity may be maintained for a considerable period (Hertlein et al., 1980, Mian and Mulla, 1983). The majority of larvicidal toxin is apparently contained within the protection of the spore wall (Myers and Yousten, 1980). However, larvicidal activity may be destroyed by high temperature (boiling 10 min.) or treatment with 0.01 N NaOH (Myers and Yousten, 1980). The effect of suspending B. sphaericus spores in unbuffered solutions ranging from pH 4.3 to 10.5 was complete deactivation of larvicidal toxins at pH above 10.0 but not at lower pH values (Mulligan et al., 1980). Karch (1982) attempted to demonstrate the effect of buffered acid and alkaline solutions on the activity of B. sphaericus but was unable to do so conclusively due to high control mortality. No investigations into the effect of pH and temperature on spore viability for prolonged periods have been reported. It was the objective of this research to investigate the interactive effect of temperature and pH on the persistence of larvicidal activity and spore viability.

MATERIALS AND METHODS
The effect of storage temperature and pH on B. sphaericus spore viability and larvicidal activity was investigated by storing spore suspensions in either citrate, phosphate, or carbonate buffers at 4° and 21°C. The buffered solutions were made according to the procedures outlined in Geigy Scientific Tables (Diehl and Lenzer, 1970). The citrate buffer (0.1 M) provided solutions with pH values of 3, 4, and 5. Three phosphate buffers (0.07 M) were adjusted to pH values in the neutral range (6, 7, and 8) and the carbonate buffers (0.1 M) were adjusted to pH 9.2, 10.0, and 10.8. Ninety-five ml of each of the nine buffers were added to each of four flasks, autoclaved (15 psi, 240°C, 20 min.) and allowed to cool before measuring the pH of each with a Beckman Zeromatic SS-3 pH meter. Five ml of a stock suspension (100 mg/l) of a lyophilized preparation (RB-80) of the 1593 isolate of B. sphaericus was used to inoculate each of the flasks. Two flasks of each inoculated buffer were stored at 4°C and the remaining two were held at room temperature (21°C).

A 10 ml aliquot of spore suspension from each of the pH-temperature regimes was serially diluted to 0.006 mg of RB-80/ml (0.006 ppm) and bioassayed against laboratory reared 48 hour 2nd instar Culex quinquefasciatus Say using the procedures outlined in Lacey (1984). Briefly, these consisted of exposing larvae to the respective treatments in waxed paper cups in 100 ml of deionized water. Twenty larvae/cup and three cups/pH-temperature regime and control were used. After adding the diluted inoculum, 1 ml of a 5 percent suspension of debittered brewer's yeast (50 mg) was added to each cup. The cups were covered with petri plates to avoid water loss and placed in trays (50 cm X 38 cm X 7.5 cm) on thermostatically controlled heat tapes (Dame et al., 1978) at 27°C for 48 hours, after which time mortality was determined.

Initially, controls were treated with citrate (pH 3) and carbonate (10.8) buffers that were serially diluted in an identical manner to those that had been inoculated with B. sphaericus. The pH in these controls was near neutral and mortality was negligible (0-2%). Deionized water (plus larval diet) was used for subsequent controls. Bioassays were begun after the spores had been held in the buffers for one week and conducted weekly thereafter until four weeks had elapsed. After four weeks, bioassays were run biweekly or until larvicidal activity had ceased. Monthly bioassays were conducted after 21 weeks until the test was terminated at week 44.

Counts of viable spores were made concomitantly with the bioassays. For each pH-temperature regime a 5 ml sample was pasteurized (80°C, 12 min.), serially diluted, and plated using the pour plate method and a selective medium (nutrient agar, 0.05% yeast extract.

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Figure 1. Larvicidal activity of *Bacillus sphaericus* suspensions held at 4°C in buffered solutions ranging from pH 3 to 10.

Figure 2. Larvicidal activity of *Bacillus sphaericus* suspensions held at 21°C in buffered solutions ranging from pH 3 to 10.
Figure 3. Viability of *Bacillus sphaericus* spores held at 4°C in buffered solutions ranging from pH 3 to 10.8.

Figure 4. Viability of *Bacillus sphaericus* spores held at 21°C in buffered solutions ranging from pH 3 to 10.8.
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and 0.01% streptomycin sulfate) as described by Yousten et al., (1982). The plates were incubated at 35°C for 48 hours and then counted.

Mortality and viable spore count data for each of the storage temperatures and pH treatments were analyzed using the SAS general linear models procedure. Regression analysis, analysis of variance, and Duncan's multiple range test were performed on all data after correcting for control mortality with Abbott's formula and arcsine transformation when appropriate. Due to day-to-day fluctuation in larval susceptibility, three test running means of mortality were utilized for analysis of mortality data.

RESULTS

The effect of pH on larvicidal activity of B. sphaericus suspensions held at 4° and 21°C for variable lengths of time is presented in Figures 1 and 2. To avoid confusion, selected graph lines that were not significantly different from adjacent lines have been omitted. There was a negative correlation between increasing pH and larvicidal activity (R = 0.76 at 4°C, slope = -5.97; 0.79 at 21°C, slope = -6.19). Complete loss of larvicidal activity was observed with spores stored at pH 10.8 within the first week at both temperatures. A sharp and significant difference in larvicidal activity (P < 0.01) is apparent between spores held under the two temperature regimes. Within the 4°C group, the spores held at pH 3-5 were significantly more larvicidal than those held at higher pH (P < 0.05). Spores held at pH 7 and 8 were not significantly different from one another. No significant difference was found between larvicidal activity of spores held at pH 6, 8, and 9.2. Those held at pH 10 were significantly less larvicidal than the rest. At the termination of the bioassay testing (308 days), spores that had been held in acid and neutral regimes still retained larvicidal activity that was close to the levels observed after the first week. In the 21°C group, only the spores held at pH 10 and 10.8 were significantly different from those held under less alkaline conditions in terms of larvicidal activity, although those held at pH 5-7 retained some larvicidal activity for a longer period of time than those held below pH 5 and above pH 7. At the termination of testing for the 21°C group (35 weeks), only negligible larvicidal activity was apparent.

Figures 3 and 4 depict the effect of pH on spore viability for suspensions held at 4° and 21°C. Viable spore counts (spores/mg RB-80) were significantly different (P < 0.001) for the two groups. Under both temperatures, there is a pronounced bactericidal effect of storage at pH 3 and 4. Excellent spore survival was observed in the 4°C group for all suspensions stored in buffers above pH 4. Although spores remained viable at pH 3 when held at 4°C, there was a rapid loss in viability at pH 5 after the tenth week of storage at 21°C. A significant steady decline in viable spores was recorded for all other suspensions stored at the higher temperature. Under both temperatures, storage in a buffer at or near neutrality is significantly better (P < 0.05) in terms of spore survival than storage at the acid or alkaline extremes.

DISCUSSION

In the studies conducted by Mulligan et al., (1980) and Karch (1982), spores held in unbuffered and buffered acid or base solutions were bioassayed against mosquitoes in the solutions in which they had been held. The results obtained were not completely conclusive, more due to the effects of the various pH extremes on the mosquitoes than to the effect of pH on the spores. Storage of B. sphaericus in the buffered pH solutions with subsequent serial dilution of the suspensions to attain the desired concentration of 0.006 mg RB-80/l in the bioassay cups allowed us to observe the effects of storage pH on subsequent larvicidal activity without the adverse effect of exposing larvae to extremes of pH, which can inhibit normal larval feeding and increase control mortality.

The data reveal an interesting relationship between pH, spore viability, and larvicidal activity. While the latter is most severely reduced by high pH, spore viability is more affected by storage in buffers at low pH. Higher temperatures exacerbate both phenomena. It is apparent that viable spores are not required for larvicidal activity. Although the denaturation of the larvicidal toxin due to storage in alkaline buffers does not reduce spore viability, however, it may inhibit the ability of B. sphaericus to recycle in mosquito cadavers under natural conditions. Prolonged persistence of both larvicidal and spore activity in the environment will optimize the propensity for the recycling of B. sphaericus.

In nature, habitats that may be extremely acid (peat bogs) or alkaline (certain desert ponds) may be uninhabitable by mosquitoes until they are inundated with fresh water during the precipitation cycle. Spores applied for mosquito control during these periods will not be subjected to the same environmental conditions they would encounter during that portion of the cycle when mosquitoes are unable to breed because of extremes in pH, temperature, or other inhospitable parameters. The ability of the spores to persist for prolonged periods in acid or alkaline environments may permit residual larvicidal control or recycling when those habitats are once again suitable for mosquito breeding.

Our data on storage in neutral buffers at 4° and 21°C essentially agree with those of Mian and Mulla (1983) up to week 14. At this point a sharp decline in larvicidal activity was observed in our bioassays for spores stored at 21°C. In their study, there was no difference in activity in spores held in tap water at room temperature and in the refrigerator for 20 weeks.

In future studies on the production and formulation of B. sphaericus, attention should be given not only to the long-term effect of drenches on efficacy but also on spore viability. Liquid formulations with pH of 6 to 8 stored at lower temperatures will ensure longevity of both spores and larvicidal toxin.

ACKNOWLEDGEMENTS

The author is grateful to Dr. Larget-Theiry, Pasteur Institute, Paris, for providing samples of RB-80. Ms. Loretta Callen provided technical assistance. Review of this manuscript by Dr. M. S. Mulla, University of California, Riverside, and Drs. A. H. Undeen and D. L. Bailey, USDA, ARS, is gratefully appreciated. This research was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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ANALYSIS OF EGG MORTALITY IN EXPERIMENTAL POPULATIONS OF TRIATOMA INFESTANS UNDER NATURAL CLIMATIC CONDITIONS IN ARGENTINA

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ABSTRACT. Seasonal changes in fecundity and egg mortality of Triatoma infestans populations were studied over three years in specially constructed chicken houses in an endemic area of the Argentine chaco. At monthly intervals the chicken houses were dismantled and rebuilt, and the bug populations subjected to a complete census. Total egg production showed marked seasonality, with peak output during the summer months. This coincided with peak recruitment of females to the population. In general, egg production was higher in bug populations with four chickens as hosts than from those with two chickens. Mean egg mortality also followed a seasonal pattern, with most mortality attributed to climatic effects during the cold winter months. Hymenopteran egg parasitoids (Teleonoma farii) began to appear towards the end of the summer and attacked up to 37 percent of eggs. Egg parasitism then declined and was not apparent during the winter. Our analysis did not reveal any density-dependence either in overall egg mortality or in mortality attributable to egg parasitoids, predators, or climatic effects.

INTRODUCTION

Triatoma infestans (Klug) is the most important and widespread vector of Chagas' disease, widely distributed throughout Argentina and neighboring countries (Fig. 1). Field and laboratory studies in many countries have contributed to our understanding of the biology of T. infestans, but there have been no long-term studies of the population dynamics of this vector under natural conditions in endemic areas. Such studies are necessary to understand natural regulatory processes within the domestic vector populations, which in turn provide valuable insight into control strategies directed against them.

Field work conducted by ourselves and others revealed considerable difficulties in the long-term study of domestic T. infestans under natural conditions, of which the following are among the most important:

1. Most sampling methods for bug populations in naturally infested houses are extremely inefficient and of poor accuracy (Leal, 1975; Pinchin et al., 1981; Schofield, 1978). Only complete demolition of infested houses and careful recovery of most of the bug populations has been found to give satisfactory data on bug population size and age structure (Marsden et al., 1979; Minter, 1977; Rabinovich et al., 1979; Schofield, 1978, 1980b).

2. Due to the long development cycle (180 days) of T. infestans populations, trends can only be determined over several generations (Rabinovich 1972; Schofield, 1980a; Szmilewicz, 1969, 1976).

3. Regular sampling of domestic bug populations over a long period often causes inaccuracy to the households. Sampling methods relying on mark-release-recapture techniques pose ethical questions by the release of vectors into a house and many householders are understandably reluctant to have such methods used (Leal, 1976; Schofield, 1978).

4. Long term studies and the need for adequate replication leads to relatively high operating costs, particularly since naturally infested houses tend to be widely dispersed (Bucher and Schofield, 1981).

In view of the difficulties inherent in the long-term study of naturally infested houses, an alternative approach has been to use physical models of the domestic ecotope in the form of artificial chicken houses which can be dismantled at intervals to observe the development of the bug infestation. This approach was pioneered by Forattini et al., (1971, 1977) for studies of the dispersal and colonization rates of T. sordida and Panstrongylus megistus in the state of Sao Paulo, Brazil, and has also been used by Espinola et al., (1979) and by Minter and Oswald (1982) in studies of Rhodnius proliscus in Venezuela. We have used a similar approach to study the population dynamics of T. infestans over a three year period in an endemic area of the Argentine chaco. In Argentina, T. infestans is frequently found in peri-domestic habitats such as chicken houses and goat corrals (Ronderos et al., 1980; Soler et al., 1977) and so the use of artificial chicken houses for the long-term study of T. infestans populations is particularly appropriate.

One objective of our study has been to determine key mortality factors throughout the climatic seasons, and to identify factors which act in a density-dependent manner on the bug populations. In this paper we report on seasonal changes in oviposition rates and sources of egg mortality. Of particular interest is egg mortality due to microhymenopteran egg parasitoids which have attracted considerable attention as possible biological control agents (Feliciangeli, 1976). The best known of

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Figure 1. Geographic distribution of *Triatoma infestans* in South America.
these, Telenomus farai Costa Lima, has been intensively studied in the laboratory by Rabinovich and colleagues (e.g. Rabinovich, 1970a, b; 1971a, b). Computer simulations (Rabinovich, 1971c, 1984) and field trials in Venezuela against *Rhodnius prolixus* (Rabinovich et al., 1980) indicate that vector control by this method would be uncertain. A survey of natural enemies of *T. infestans* in the province of Córdoba, Argentina, where our work was carried out, revealed seven species of microhymenopteran egg parasites: *Anastatus catamaracensis* (Brethes), *A. charitos* De Santis, *A. coreophasus* Ashamed, *A. excavatus* De Santis, *Oolothon mireyae* De Santis, *Ooencyrtus venatorius* De Santis and Vidal, and *T. farai* (Brewer et al., 1984a). Experimental release of the last species was not successful, however, in controlling domestic populations of *T. infestans*, (Brewer et al., 1982). Other natural predators of *T. infestans* eggs include ants, crickets and spiders (Barrett, 1976; De Santis et al., 1981).

**MATERIALS AND METHODS**

**Experimental Chicken Houses**

Our studies were carried out on populations of *T. infestans* maintained in experimental chicken houses near the village of Huascha, Department of Cruz del Eje, Province of Córdoba, Argentina. Construction of the chicken houses mimicked naturally infested chicken houses in Córdoba Province where Chagas’ disease is highly endemic.

The chicken houses were built of locally made adobe bricks, without mortar, in such a way that they could be easily dismantled and rebuilt each month (Fig. 2). In this way, all the bugs infesting each chicken house could be counted, identified to stage, and measured before being returned to the same chicken house to continue their development. Each chicken house measured 100 × 80 × 80 cm, and was divided internally by a horizontal layer of wire-netting, 50 cm from the base, which provided space for the chickens below and supported cardboard slats above as refuges for the bugs. A hardboard cover formed the roof, and each chicken house was mounted on a smooth cement base to facilitate cleaning and collection of fallen bug eggs.

To prevent bugs escaping and to prevent the entry of wild (possibly infected) bugs or undesired animal intruders, the chicken houses were sited within a wire-mesh compound, and each chicken house was covered by an individual cover of plastic mosquito netting on a wooden frame. The chickens were allowed to forage within the compound during the day but were replaced in the chicken house each night.

Maximum and minimum temperatures and total rainfall were recorded daily at the site (Fig. 3).

**Experimental Procedure**

The results reported in this paper refer to six experimental chicken houses, three of which were set up each with two chickens as hosts, and three with four chickens each. Each chicken house was colonized in March 1981, with 300 eggs, nymphs, and adults of *T. infestans* representing all developmental stages in proportions following a calculated stable age distribution (Rabinovich, 1972). All bugs, except eggs and first-stage nymphs, were individually marked with acrylic paint on the legs. The *T. infestans* originated from a stock colony derived from the offspring of recently collected bugs from localities within the Province of Córdoba.

For three years, at approximately monthly intervals, the six chicken houses were dismantled and rebuilt, and a complete census of all bugs was taken. Fertile eggs were returned to the chicken house, along with live nymphs and adults, to complete their development. Eggshells and dead eggs were removed for further examination. During each census the presence of potential egg predators was noted and sample specimens removed for identification. At each census, the total number of eggs and eggshells could be classified as follows:

1. Fertile, embryonated eggs. Color pearly-white to pink, according to the stage of embryonic development; with intact operculum.
2. Infertile or dead eggs. Uneven, collapsed shape; color uneven, violet, whitish, or yellowish; with intact operculum. Cause of egg failure unknown, but could include infertility and climatic effects.
3. Parasitized eggs. Color grayish, indicating presence of parasitoids and their remains inside. Operculum intact, but sometimes a small, ragged parasitoid exit hole, usually opposite the operculum. Intact parasitized eggs were maintained in the laboratory at about 26°C and 60 percent relative humidity for identification of emerging parasitoids.
4. Damaged eggs, with evidence of attack by arthropods with biting and chewing mouthparts.
5. Eggshells. Ecloded eggs without operculum, matt white in color. Represented successful emergence of a first-instar *T. infestans* nymph. Chorions were found to be very light and could be blown away by the slightest breeze, suggesting that some might have been lost during collection. As a check on this, the number of eggshells collected at each census was compared with the number of first-instar nymphs collected and corrected accordingly.

**RESULTS**

Total egg production from the six chicken houses showed marked seasonality, with peak output during the summer months (September-March) (Fig. 4). This coincided with peak recruitment of females to the population (Fig. 5) while maximum egg output per female coincided with the onset of peak egg production (Fig. 6). Egg output from chicken houses with four chickens was higher than those with two chickens since the higher host density in general maintained larger populations of bugs, until December 1982, when there was a high mortality of nymphs and adults in chicken houses with four chickens. However, although bug density in chicken houses with four chickens dropped to low levels, egg output was similar between populations with two and four chickens during the following winter. Bugs with more hosts as food source recovered their preceding density levels and egg output at the next hot season (Fig. 3).

Average egg output per female per month during the summer months was 140-160 compared to 10-60 during the winter months. This compares with averages of 39-105 (smallest) (Rabinovich, 1970a), 15-38 (Rabinovich, 1979) for female *T. infestans* maintained in laboratory colonies.

Mean egg mortality also followed a seasonal pattern but with considerable variation about the mean (Figs. 7 & 8). Strong positive correlation was found between the mean mortality and its variance (TABLE 1), which could suggest a degree of heterogeneity in egg survivorship.
Figure 2. Structure of the chicken house used to study *Triatoma infestans* populations. a - adobe bricks, b - wooden frame, c - mosquito netting, d - hardboard cover, e - cardboard slats, f - space for chickens.

Figure 3. Climatic records at field site. Mean maximum and minimum temperatures (upper and lower lines) and monthly rainfall (histogram).
TABLE 1. Analysis of correlation between log (mean egg mortality per month) and log (standard deviation).

<table>
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$r =$ linear correlation coefficient  
$b =$ slope  
$F2 =$ parasitoids  
$F3 =$ predators  
$F1 =$ climate and infertility

TABLE 2. Analysis of key factors for egg mortality. Key as in TABLE 1.

| Density of Host Chickens | Replicate | No. of Censuses | Source of Mortality |  |  |  |  |  |
|--------------------------|-----------|----------------|---------------------|---|---|---|---|
|                          |           |                | F1      | b        | F2      | b        | F3      | b        |
| 2                        | 1         | 20             | .046    | .012     | .749    | .647     | .501    | .341     |
|                          | 2         | 24             | .995    | 1.015    | -.084   | .008     | -.129   | -.007    |
|                          | 3         | 24             | .924    | .895     | .286    | .110     | -.069   | -.005    |
|                          |           |                | Total    | 68       | .875    | .811     | .351    | .156     | .139     | .032     |
| 4                        | 1         | 26             | .859    | .683     | .214    | .066     | .679    | .251     |
|                          | 2         | 21             | .642    | .469     | -.050   | -.008    | .691    | .538     |
|                          | 3         | 22             | .324    | .151     | .857    | .797     | .182    | .052     |
|                          |           |                | Total    | 69       | .658    | .465     | .389    | .193     | .579     | .342     |

TABLE 3. Analysis of density-dependence of egg mortality. Key as in TABLE 1.

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<th>No. of Censuses</th>
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Figure 4. Mean total *Triatoma infestans* eggs in chicken houses with two (-----) and four host chickens (———).

Figure 5. Mean female recruitment in chicken houses with two (-----) and four host chickens (———).

Figure 6. Mean fecundity (No. eggs/female/month) in chicken houses with two (-----) and four host chickens (———).
Figure 7. Mean egg mortality in chicken houses with four host chickens. A - total mortality, expressed as proportion or total eggs, B - mortality attributed to climate and infertility, C - mortality attributed to parasitoids, D - mortality attributed to predators. (B, C, D, expressed as proportion of total mortality.)
Figure 8. Mean egg mortality in chicken houses with two host chickens. A - total mortality, expressed as proportion of total eggs, B - mortality attributed to climate and infertility, C - mortality attributed to parasitoids, D - mortality attributed to predators. (B, C, D, expressed as proportions of total mortality.)
Causes of egg mortality were determined as follows:

F1. Mortality due to climatic effects and/or infertility.
F2. Mortality due to hymenopteran egg parasitoids.
F3. Mortality due to biting and chewing predators.

Strong positive correlation was found between the mean and variance of mortality attributable to each of these factors.

Mortality due to climatic effects and infertility reached maximum values, up to 50 percent, during the winter season (June-September), whereas at other times it rarely reached more than 5 percent (Figs. 7 & 8). In laboratory colonies of T. infestans, the proportion of infertile eggs rarely exceeds 5 percent (Zrumalewicz, 1969; Rabinovich, 1972), so it is reasonable to suggest that similar rates of infertility were acting on our bug populations, with the remaining mortality in this category attributable to low temperature.

Egg parasitoids began to appear towards the end of the summer (February-March) and attacked a high proportion of the eggs, particularly during the 1982 summer when egg parasitism in the chicken houses with four hosts reached 37 percent and 18 percent for those with two hosts. Egg parasitism then declined and was not apparent during the winter months (Figs. 7 & 8). The structure of the exit holes of the parasitized eggs corresponded to those due to T. farinii, and this was the only species which emerged from parasitized eggs taken to the laboratory.

Mortality due to predators was highly variable between years and among chicken houses, and reached high levels only occasionally during the height of the summer (November-December) (Figs. 7 & 8). The pattern of predatory action was slightly higher in chicken houses with four chickens (see below).

Key Factor Analysis

Key factor analysis of egg mortality followed Podoler and Rogers (1975) by comparing log mortality attributed to each specified factor (k1) to the total egg mortality (K) for each census. The k-factor for each source of mortality was calculated as k1 = log (1-no. dead by source / total eggs).

By this method, the slope of the regression is proportional to the importance of the factor in contributing to the total mortality. Separate analysis of each replicate gave no clear indication of a key factor, since each factor predominated in at least one replicate. However, grouping the three replicates for high and low host densities clearly showed that mortality due to climatic effects was the most significant (TABLE 2). Mortality due to egg parasitoids was of similar importance at both densities of host chickens, but mortality due to predators was more important in chicken houses with four chickens.

Analysis of Density-Dependence

Analysis of density-dependence in the three sources of egg mortality followed Varley and Gradwell (1960), by comparing the k-factor for each source of mortality with the log total egg numbers at each census. This comparison was made by linear regression (TABLE 3), and by sequential graphs to detect delayed density-dependence. Neither separate analysis of each replicate nor analysis of replicates grouped according to host density revealed any indication of density-dependence since the slope of the regression did not differ significantly from zero. Sequential graphical analysis supported this conclusion following a density-independent pattern for each source of mortality.

Discussion

Physical models of the natural habitats of triatomine bugs are powerful tools for the study of colonization rates (Forattini et al., 1971, 1977), population dynamics (Espinola et al., 1979; Gorla, 1984, Gorla and Schofield, 1983), and potential control methods (Minter and Oswald, 1982). Our studies using experimental chicken houses in Argentina have provided substantial data on the population dynamics of T. infestans, which are to be reported in detail elsewhere.

The present work has analyzed three important sources of egg mortality in T. infestans populations under natural climatic conditions. The strong positive correlation between the mean and variance of egg mortality both overall and for each source of mortality considered separately, indicates a degree of heterogeneity in the egg survivorship. One interpretation of this could be greater exposure to causes of mortality at higher egg density, but subsequent analysis did not reveal density-dependence in any of the three factors contributing to egg mortality.

In the Argentine chaco, where our study was sited, mortality attributable to climatic effects was more important than the action of egg parasitoids or predators. This is no doubt related to the low nighttime temperature during the winter months, but the overall effect is balanced by the fact that this is also the period of lowest egg production.

The main period of oviposition extended throughout the summer from September to March, declining rapidly with the onset of winter. Egg parasitism increased only towards the end of the oviposition period and declines to zero during the winter months when few eggs were present. This highlights the weakness of biological control using egg parasitoids (Brewer et al., 1982, 1984b; Rabinovich et al., 1980), since their action is limited to periods of high egg production. Thus, although it seems likely that the density of egg parasitoids would be limited by the density of triatomine eggs, our study showed no evidence that egg mortality was dependent on the density of egg parasitoids.

Egg mortality due to predators was generally low, especially in chicken houses with only two host chickens. Egg predation often peaked during November-December when total egg production was high, but no density-dependence in mortality due to predators was revealed by our analyses. The general absence of density-dependent egg mortality shown in our study supports the view of Schofield (1982, 1985) that density-dependent mechanisms in T. infestans act principally on the birth rate rather than on the death rate. Our results suggest that neither egg parasitoids nor predators would be effective as biological control agents against T. infestans because of their low effectiveness and insensitivity to changes in the density of their hosts.
Acknowledgements

We thank Dr. Ken France, Proyecto Bicudo, Cruz del Eje, for his help in the initial stages of this work. Financial support from CONICET, Buenos Aires, and from the Wellcome Trust, London, is gratefully acknowledged.

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A POSSIBLE HUMAN INFESTATION BY
EUTROMBICULA BELKINI (GOULD) (ACARI: TROMBICULIDAE)
IN LAGUNA BEACH, CALIFORNIA

Stephen G. Bennett† and James P. Webb, Jr.

ABSTRACT. Numerous larval Eutrombicula belkini were recovered from the grounds near a residence in Laguna Beach where a 40-year-old woman and a 52-year-old man had experienced numerous pruritic bites. The lesions were dispersed in patterns that correlated with typical trombiculid mite infestation patterns on humans. The premises were treated with chlorpyrifos (Dursban® 2E) after which no further dermatological problems were noticed.

INTRODUCTION

Larval trombiculid mites (chiggers) are common in the United States and several species are known to attack humans in the midwest and southeast. Few reports of chigger mite attacks involving people have been reported from southern California. When recorded, Eutrombicula species are usually responsible for the painful itching and discomfort in human hosts and have been recorded by Doetschman and Purman (1949), Gould (1950, 1956), Bennett (1977), Bennett and Loomis (1980), and Webb et al. (1983).

A man and his wife (Mr. & Mrs. R.) moved into their Laguna Beach home on June 16, 1985. The previous morning she and Mr. R. had watered their yard and pulled weeds from alongside of their house in the afternoon. Mrs. R. noticed small "red spots" on her leg early in the afternoon of June 25, that began to severely itch that night. The next day she again worked in the yard and the following day she had additional red spots on her back, shoulder (underneath bathing suit strap), neck, chin, and right hip (under suit strap). On June 28, she noticed more spots on her neck, ear, and eye lid. Mr. R. worked all day in the yard and on the hillside near the woodshed above the house on June 29, and noticed itchy red spots on his arms and legs on June 30.

Mr. and Mrs. R. described the bites as follows: Small reddish blotches with a tiny red dot in the center that did not itch initially. After itching commenced, scratching caused a stinging sensation to become more intense and the bites became inflamed and formed fluid-filled blisters. Eventually, the blisters broke and formed a crusty covering over the bites on the skin. The bites were most numerous beneath bathing suit straps on the torso (Fig. 1) and on the legs.

On July 1, the Orange County Vector Control District (OCVCD) was contacted by Mrs. R. who inquired about the possibility of chiggers attacking them in their yard because both she and her husband were suffering from small, red lesions that were severely itching. In addition, she related the information that she had chigger bites while living in Wisconsin and the current symptoms were similar. She was not aware, however, that chiggers occurred in California.

MATERIALS AND METHODS

Mr. and Mrs. R. were advised to place a dark colored plastic plate or some other dark, flat object on the ground where they were working. This technique was described by Gould (1956), Loomis (1956), and Bennett (1977) and proved to be quite successful in collecting chiggers if placed out during peak activity periods.

On July 2, OCVCD personnel placed black plates on the ground at 10:30 A.M. in areas where Mr. and Mrs. R.

† Orange County Vector Control District, P.O. Box 87, Santa Ana, CA 92702, U.S.A.
had been working (Fig. 2). The plates were checked every few minutes for the presence of chiggers until 11:30 A.M. In addition, three western fence lizards (Sce1oporus occidentalis) were noosed beside the house and were checked for chiggers on the neck and legs. Two plates, alcohol, and instructions for collecting unfed chigger larvae were left with Mr. and Mrs. R. They were advised to collect earlier in the morning to avoid warmer conditions later in the day. OCVCD personnel returned on July 9, and set out black plates at 9:30 A.M. underneath Artemesia californica (coastal sagebrush) and Eriogonum sp. (buckwheat) that were growing near a woodshed situated on a slope above the house (Figs. 4 & 5). Plates were also placed beneath several small ornamental trees and among some dense weeds. An additional western fence lizard was also collected during the course of this surveillance.

![Figure 2. Black plastic plate beneath Artemesia californica (coastal sagebrush) used to collect unfed Eutrombicula belkini larvae.](image)

**RESULTS AND DISCUSSION**

No chiggers were recovered between July 1-8, although Mrs. R. observed a small reddish mite crawling across a piece of paper she placed on the ground on July 1. However, the specimen was not preserved. Chiggers were not seen on black plates by OCVCD personnel or on any captured fence lizards during surveillance work on July 2. The plates placed between 10:00 and 10:30 A.M. on July 9, under coastal sagebrush yielded six reddish, rapidly moving mites. Air temperature was approximately 80°F and relative humidity was high. No mites were recovered from the other sites. Three of the retrieved mites were mounted on microscope slides in polyvinyl alcohol-lactic acid (PVA-L) and identified as unfed larvae of *Eutrombicula belkini*, a known human pest chigger (Fig. 3). The other three specimens were lost in transit.

![Figure 3. Unengorged Eutrombicula belkini larva (X100).](image)

More mites may have been collected if sampling were done earlier in the morning (6:00-8:00 A.M.) before the ambient temperature rose. Gould (1956) mentioned that activity of *E. belkini* observed in Palos Verdes Estates (Los Angeles Co., CA) was greatest before the morning fog burned off and by noon "it apparently became so warm as to drive the chiggers to shelter." Gould (1956) and Webb et al., (1983) described the areas having the greatest concentrations of chiggers as zones composed of *Artemesia californica* and annual grasses, with the highest number of specimens being collected from near the bases of sagebrush.

The finding of *Eutrombicula belkini* specimens at the Laguna Beach residence does not necessarily prove that they were the cause of the reported skin irritations. No chiggers were recovered from the area where most of the gardening activities occurred and no specimens were retrieved from the skin of the affected individuals. However, Mr. R. had worked on the slope near the woodshed where chiggers had been collected.

Well-watered lawns have been identified as sources of chigger infestations in California. The tropical pest chigger (*Eutrombicula batatas*) was collected from the lawns at a park and several residences in Bakersfield (Doetschman and Furman, 1949). Bennett and Loomis (1980) reported *E. batatas* and *Eutrombicula alfrededagensi* from Pima County, Arizona during August after summer rainfall, and *E. batatas* from moist environments near irrigation ditches and the Colorado River. Bennett (1977) also noted the recovery of several species of
Eutrombicula from southern Arizona (including E. belkini) after summer rainfall in the arid foothills and higher mountains.

Webb et al., (1983) reported the relatively high prevalence of sagebrush-grassland ecotones in Ventura County, especially around agriculture and housing tracts, which seemed to favor the productivity of large E. belkini numbers when conditions were optimal. These same ecological and environmental conditions may explain the occurrence of E. belkini at the Laguna Beach residence.

A cursory query of neighbors by Mr. and Mrs. R. revealed that other people in the vicinity had apparently not been similarly affected. The immediate environs adjacent to the Laguna Beach house were treated with chlorpyrifos (Dursban® 2E) on July 9 by a private pest control company. No further complaints regarding skin irritation problems have been received since.

Figure 4. Coastal Sagebrush/grass ecotone. a - Artemesia californica; b - Location of black plate.

Figure 5. Hillside adjacent to Laguna Beach residence. a - Location of unfed Eutrombicula belkini larvae.
Acknowledgements

We thank Gilbert L. Challet, Manager, Orange County Vector Control District for the use of the laboratory facilities.

REFERENCES CITED


DERMATOLOGICAL REACTIONS TO THE BITES OF FOUR SPECIES OF TRIATOMINAE (HEMIPTERA: REDUVIIDAE) AND CIMEX LECTULARIUS L. (HEMIPTERA: CICICIDAE)

Raymond E. Ryckman

Hypersensitivity reactions of varying degrees that result from the bites of Triatominae and Cimicidae have been documented in humans by many researchers (Ryckman, 1979; Ryckman and Bentley, 1979). Additional information may be obtained by consulting Costa et al., 1981; Edwards and Lynch, 1984; Goldman, 1971; Marshall and Street, 1982; Marshall, 1982; and Pinnas et al., 1978.

Most of the many observations have been of symptoms that follow a bug bite, but some were based upon suspect rather than positively identified arthropod species; controlled experimental conditions were uncommon. Experiments with human subjects are self-limiting because of ethical practices, incomplete health records of individuals, and sparse data on the genetic history of each person. In spite of these shortcomings, any information accrued from empirical observations still provides valuable baseline data for arthropod/dermatological reaction studies. In this context, the following is reported from observations of bite reactions induced by Triatoma protracta, Triatoma lectularia, Triatoma dimidiata, and Dipetalogaster maxima in 481 adult human subjects. An additional 14 subjects were monitored after being bitten by Cimex lectularius.

MATERIALS AND METHODS

The subjects in this study were 495 young adults (21-30 years old) who had no known history of exposure to the bites of Triatominae spp. or Cimex spp. Unfed Triatoma spp. and Cimex lectularius were placed (one per vial) in small, cloth-covered, plastic vials. A piece of blotter paper was inserted in each vial to enable the bugs to climb up to the exposed forearm of each person. The vials were held firmly against the forearm with adhesive tape, and the bugs were then allowed to feed to repletion through the cloth cover on the end of the vials. Records of localized skin reactions were made immediately following the bite and at 2, 24, 48, and 72 hours post bite. Persons with a history of hypersensitivity to insect stings or food allergies were not tested. Each bite reaction was based on the bite of one insect.

The records of the bites were subjectively based upon the degree of dermal pruritus, erythema, and edema at stated intervals post bite.

RESULTS AND DISCUSSION

A total of 2.49 percent (12 persons of 481) demonstrated one or more types of skin reaction to Triatominae bites, and 21.43 percent (3 persons of 14) demonstrated one or more types of skin reaction to bed bug bites.

Most of the skin reactions (pruritus, edema, and erythema) were not severe (see TABLES 1 and 2). These levels of response were expected because the subjects had no history of exposure to Triatominae bites. From an immunological point of view one would not have expected any reactions in persons previously unexposed to the bites of these bugs. The following suggestions serve as a possible explanation of the results and should be considered in evaluating these data.

1. The persons in this report were relatively young adults (21-30 years of age) who indicated they had not previously been bitten by Triatominae or Cimex species. However, their traveling and camping in the mountains or deserts of southwestern United States may have exposed some of them to bites that occurred without their knowledge. Hence, they could have been previously sensitized.

2. The observed reactions to the bites may have been cross-reactions due to previous bites by other species of biting insects. Such cross-reactions are even possible in monoclonal antibody systems. Unexpected cross-reactions as cited by Lane and Koprowski (1982) are uncommon, but they can and do occur (Goding, 1983: 43). 

3. Of the approximately 20 subfamilies of the family Reduviididae, 19 subfamilies are predaceous and capable of inflicting a very painful bite associated with the injection of highly proteolytic enzymes, which rapidly break down the proteins of the prey. The exception to the above is, of course, the species of the subfamily Triatominae that are hemophagous on vertebrates and characterized by possessing a relatively benign bite. The saliva, injected when these bugs feed, functions as an anticoagulant and does not break down host tissues. The hemophagous nature and benign bite of the Triatominae would appear to be a secondarily derived phylogenetic condition. It is, therefore, proposed that minute traces of predaceous reduviid-like venom may be present in some Triatominae bugs and thus elicit unexpected responses.

In conclusion, it is the opinion of the writer that the reactions observed were due to the possible causes expressed in numbers 1. and/or 2., more probably a combination of both. Additional studies are strongly encouraged.

1 Department of Microbiology, School of Medicine, Loma Linda University, Loma Linda, California 92350 U.S.A.
TABLE 1. Percentage of persons reacting to bites of Triatominae and Cimicidae. A summary of the more involved reactions.

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**PRURITUS**

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*These calculations were based on 72 subjects rather than 93.

TABLE 2. The percentage of persons reacting to bites of Triatominae and Cimicidae.

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TABLE 2.  (continued)

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Acknowledgements

Cristina M. Blankenship and Carol D. Stringer critically read the manuscript. William Eby, M.D. and Giuseppe Molinaro, M.D. were consulted on immunological aspects of this problem. The four persons mentioned above are members of the staff or faculty of the Department of Microbiology of Loma Linda University.

REFERENCES CITED


CORRIGENDUM

An omission error occurred in the first printing (BSOVE 10(1): 70) of the following article. It is presented here in its corrected form along with our apology to Dr. Elbel.

ECOLOGY AND SYSTEMATICS OF SOME NEARCTIC LEPORID FLEA LARVAE\(^1\)

Robert E. Elbel\(^2\)

ABSTRACT. Cediopsylla simplex is known to be dependent on the reproductive cycle of its host and eggs are laid in the nest following copulation on newborn nestlings. Larvae were collected by Nixon Wilson (University of Northern Iowa, Cedar Falls, IA) from nests of Sylvilagus floridanus that yielded only C. simplex adults; no other true rabbit flea is found in the immediate area. All three instars of Euhoplopsyllus g. glacialis are known to live in the fur of Lepus arcticus from May through July, the breeding season of the hare, which has no permanent nest or resting place. Larvae (of E. g. glacialis) were supplied by Bob Pilgrim, University of Canterbury, Christchurch, New Zealand. Rabbit flea larvae have a mandible with five teeth, posterior row on abdominal segments 1-6 with five setae, anterior row on abdominal segment 9 with four short setae, anal segment with three ventrolateral setae, and anal comb with a single row of setae. These characters separate rabbit flea from all others except Ctenocephalides felis and C. canis in which the anal comb has 10-13 setae instead of 8 as with Cediopsylla simplex and 5-6 as with Euhoplopsyllus g. glacialis.

\(^1\) Based upon a presentation made at the Joint Session of the Utah Mosquito Abatement Association (37th Annual Meeting) and The Society of Vector Ecologists (16th Annual Conference), 30 September through 3 October, 1984, Salt Lake City, Utah.

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