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Key Terms

- Benthic
- Brackish
- Endemic
- Ephemeral
- Vector
- Vernal Pools
- Xeric

Toxicological Responses to *Bacillus thuringiensis israelensis:* Mortality Comparisons of two Vernal Pool Inhabitants

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Abstract

From 1999 to 2002, six vernal pools were created in the University of California Natural Reserve System's San Joaquin Freshwater Marsh Reserve. The pools were inoculated with dried benthic samples from a series of vernally filled basins. Resilient populations of *Brachinecta lindahli* and other indicator species developed and have been appearing annually as the pools fill with winter rain. The pools also sustain mosquito larvae, and the application of BTI, a larvicide derived from *Bacillus thuringiensis israelensis*, was suggested. Before using BTI, however, we tested its impact on *B. lindahli* individuals recovered from the vernal pools. BTI has been reported to have no impact on other species of fairy shrimp, but toxicity effects for *B. lindahli* had not been investigated. A sequence of nine tests was conducted in which the recommended dosage of liquid BTI was applied to microcosms containing vernal pool water and ten individuals of the fairy shrimp, mosquito larvae and other invertebrates from the pools. Our results indicate that BTI kills mosquito larvae within hours but does not appear to be toxic to *B. lindahli*. In most of the tests, no mortality occurred during the first four days after treatment, and subsequent mortality followed the general pattern of natural mortality in the controls. Thus, BTI is a safe method of selectively killing mosquito larvae in sensitive ecosystems, such as vernal pools.

Faculty Mentor

Many species of fairy shrimp that inhabit vernal pools are endangered or threatened. Mosquitoes use vernal pools, and the primary mosquito species in our pools is *Culex tarsalis*. Prior to treating sensitive habitats such as vernal pools with larvicides to reduce mosquito presence, it is prudent to determine their possible impact upon fairy shrimp and other vernal pool invertebrates. This study examined the influence of the larvicide BTI (*Bacillus thuringiensis israelensis*) upon *Brachinecta lindahli* and other invertebrates from our vernal pools. BTI had no significant effect upon the fairy shrimp or other vernal pool invertebrates at levels that quickly killed mosquito larvae. This finding allows the option of using BTI treatment to control and reduce *C. tarsalis* without concern about the fate of fairy shrimp or the other invertebrate fauna in the pools.

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Introduction

The complexity of an aquatic ecosystem allows for a broad diversity of organisms to coexist. Species that live in the aquatic ecosystem, along with terrestrial life in surrounding areas, are directly affected by water changes. Variations in water availability are caused by multiple factors, such as seasonal changes and drought (Mitsch and Gosselink, 1993). As an ephemeral aquatic ecosystem, a vernal pool experiences extreme seasonal water levels, and supports both benthic (at the bottom) and terrestrial organisms.

Vernal pools experience water fluctuations by drying up annually in the late spring until winter rains replenish them (Borgias, 2004). This temporary nature requires organisms to evolve adaptations that allow them to thrive in both the wet and xeric (dry) phases of the vernal pool's annual cycle (Broch, 1965). The pools are easily susceptible to increasingly drier conditions caused by climate change and human activity, which can eliminate the pools at a rapid rate (King, 1998). One devastating consequence of this habitat loss is the decrease in endemic species that have evolved specialized adaptations for the vernal pools.

Fairy shrimp (*Brachinecta spp.*) are an invertebrate indicator species of vernal pools, and many *Brachinecta* species have become endangered due to the loss of these habitats (Belk, 1998). These crustaceans use the seasonal characteristics of the pools to survive and reproduce. The uncertainty of water availability causes the fairy shrimp to produce dormant embryos that can last several years in the benthic sediment (Belk, 1998). As the ephemeral pools become filled, eggs from the previous wet season begin to break dormancy as the right conditions are reached (Broch, 1965). This unique adaptation allows fairy shrimp to endure seasonal dry conditions. Anthropogenic and environmental impacts that cause the pools to remain dry for longer periods during the year have directly resulted in the endangerment of fairy shrimp species, such as *Artemia salina* (Belk, 1998). One species of fairy shrimp, *Branchinecta lindahli*, however, is not endangered and is known to flourish in vernal pools in Southern California. Therefore, it is important to test the resilience of *B. lindahli* in order to prevent the species from becoming extinct.

In contrast to the declining number of fairy shrimp species, mosquitoes (*Culex tarsalis*) are abundant. These opportunistic invertebrates are capable of reproducing rapidly in vernal pools, the conditions of which make them ideal breeding habitats for several mosquito species (Crans, 2004). Mosquito larvae complete their life cycle by meta-

morphosis, eventually becoming flying terrestrial adults (Johnson and Renchie, 2007). Their ability to fly has enabled them to easily transmit blood-borne diseases such as West Nile virus, malaria, dengue fever and yellow fever (Sutherst, 2004). Due to the dangers these vector-born infectious diseases pose to human health, land managers and vector (an organism that acts as a carrier) control agencies constantly strive to develop ecologically safe methods of mosquito population control. One of the most selective methods of control focuses on the larval stage. Although past methods of vector control—including draining wetlands or applying DDT, diesel fuel, and various oils—have had some success in reducing mosquito populations, mosquitoes eventually evolved a resistance against these synthetic larvicides (Olliaro and Trigg, 1995). Furthermore, these products have been proven to harm other species and environments and, as a result, are no longer legal in the United States and many other countries (Cooke, 1972).

Recently, more environmentally benign methods of mosquito control have been developed to reduce mosquito populations in sensitive sites such as vernal pools. A widely applied, purportedly mosquito-specific and ecologically responsible method of control is the use of the bacteria *Bacillus thuringiensis israelensis*, or BTI. This biological larvicide has been found to target mosquitoes successfully without harming other species. The components of BTI cause a change in pH upon ingestion, resulting in the disruption of the epithelial lining of the larval midgut (Garcia et al., 1980), eventually leading to the death of mosquito larvae that ingest it (Rey et al., 1998). This method has been eco-toxicologically tested on a number of non-target species to ensure that it is only harmful to mosquitoes (Boisvert and Boisvert, 2000; Bravo et al., 2007). Since the application of BTI is a more recent method of vector control than the use of DDT and other harmful pesticides, fewer experiments have been performed to ensure its safety. For instance, *B. lindahli* had not yet been exposed to BTI under experimental conditions.

In the University of California Natural Reserve System's (UCNRS) San Joaquin Freshwater Marsh Reserve, *B. lindahli* and *C. tarsalis* larvae co-occur in vernal pools. The close vicinity of the reserve to humans makes the presence of mosquito larvae a concern. Before applying BTI to vernal pools containing *C. tarsalis* larvae, the toxicity of BTI was tested on *B. lindahli* to determine its eco-toxicological effect on the crustacean. Mortality was compared between mosquito larvae and *B. lindahli* individuals in an experimental setting. It was hypothesized that the suspension of aqueous BTI at recommended levels would have no effect on

B. lindahli, whereas it would cause mosquito populations to decline significantly.

Methods

Field Site and Natural History

The UCNRS San Joaquin Freshwater Marsh Reserve, in Irvine, CA, is a 202-acre brackish-water wetland with approximately 50 acres of artificially created experimental ponds. Six vernal pools were established within the reserve from 1999 to 2002, at latitude 33.66 and longitude -117.85. Dried benthic sediment samples from the UC Irvine main campus were introduced into the temporary pools, initiating the growth of several vascular plants and invertebrates (Bowler and Elvin, 2003). The inoculated vernal pools are situated within several surface depression wetland communities that serve as a refuge for a range of terrestrial and aquatic species. As a result of the inoculation, the six vernal pools were rich in both species of interest, *B. lindahli* and *C. tarsalis*, along with other invertebrates (Table 1).

Table 1

Aquatic invertebrates found in the six vernal pools in the UCNRS San Joaquin Freshwater Marsh Reserve.

Insecta
Coleoptera
Dytiscid Beetle (Family Dytiscidae)
Haliplid Beetle (Family Haliplidae)
Whirligig Beetle (Family Gyrinidae)
Diptera
Mosquitoes (Family Culicidae)
Odonata
Dragonfly (Family Libellulidae)
Plecoptera
Stonefly (Suborder Arctoperlaria)
Tricoptera
Caddisfly (Suborder Annulipalpia)
Crustacea
Anostraca
Fairy Shrimp (Family Brachinetidae)

Experimental Design

Nine experimental treatments were conducted, each with a corresponding control. Each container was filled with an aliquot of 1.5 gallons of water collected from the vernal pools. The water collected was specifically filtered for any *C. tarsalis* larvae and *B. lindahli* before adding them to the containers. The quantities of mosquitoes and fairy shrimp were standardized by placing ten of each species in each microcosm. Various other insects commonly found in the vernal pools remained in the water, but were not quantified (Table 1). One drop (0.05 mL) of BTI, the dosage recommended by manufacturers, was added to the experimental containers, but not to the control containers. All containers were stored indoors to prevent any undesired factors that might have affected the mechanism of BTI, such as UV light.

The first observations were made before any BTI was added. Twenty-four hours after exposure, the quantity of live mosquitoes and fairy shrimp remaining was noted. Subsequent observations were made roughly every 24 hours, for 96 hours. Behavior was also noted, taking into account the activity level of the organisms.

Statistics

After the initial 24 hours, the statistical significance of BTI on fairy shrimp and mosquito mortality was calculated by ANOVA using Systat statistical and graphical software. This process was repeated after 96 hours had passed to allow sufficient time for BTI to take effect. The average numbers of living fairy shrimp and mosquitoes over time were plotted in Excel to visualize mortality trends.

Results

After 24 Hours

After 24 hours, BTI did not significantly affect mortality of *B. lindahli* (P = 0.190). Rather, *B. lindahli* on average lived longer in the treatment than in the control. The mean value for the number of surviving individuals after 24 hours for the treatment was 10.00 ± 0 individuals, whereas the mean was only 9.20 ± 1.79 individuals for the control (Figure 1).

For mosquitoes, it was found that BTI had a significant affect on the ability of mosquitoes to survive (P = 0.006). The mean of surviving mosquito individuals after 24 hours was 8.60 ± 3.13 in the control, and only 2.78 ± 3.15 in the treatment (Figure 2).
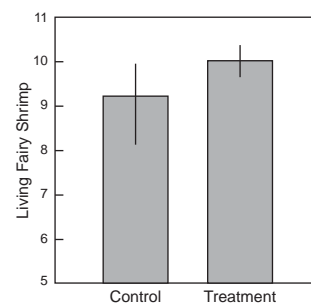
Figure 1

Average number of *B. lindahli* in both control and treatment after 24 hours had passed. Error bars represent standard error.
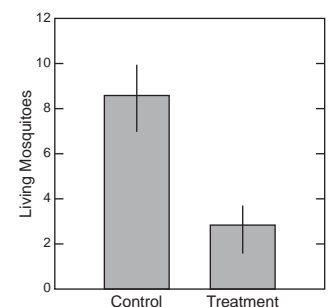
Figure 2

Average number of mosquitoes after 24 hours for both control and treatment. Error bars represent standard error.

After 96 hours

After 96 hours, the BTI was still not significantly effective in killing *B. lindahli* (P = 0.173). The mean number of living individuals after 96 hours was 4.20 ± 3.90 for the control and 6.78 ± 2.77 for the treatments (Figure 3).

It was found that after 96 hours, BTI was still significantly affecting mosquito mortality (P < 0.001). The mean number of individuals living was 5.40 ± 3.58 in the control, and no mosquitoes survived in the treatment (Figure 4).
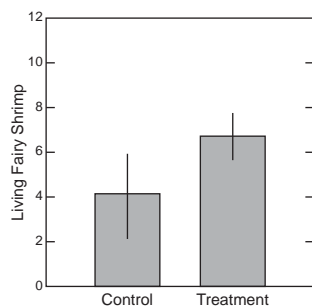
Figure 3

Average number of *B. lindahli* alive after 96 hours in both the control and treatment. Error bars represent standard error.
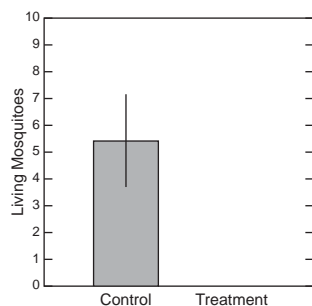
Figure 4

Average number of mosquito individuals alive after 96 hours in both the control and treatment. Error bars represent standard error.

Duration

To view the trend of mortality, averages were calculated for each of the 96 hours. On average, *B. lindahli* lived longer in treatment conditions, and mosquitoes lived longer in control conditions (Figures 5 and 6).

Discussion

Results showed that the recommended dosage of BTI significantly affected mosquito survival from 24 to 96 hours. In contrast, BTI did not significantly affect the ability of *B. lindahli* to survive; in fact, the fairy shrimp actually lived longer in the treated containers than the controls. Overall, application of BTI was toxic to mosquito larvae, but did not harm coexisting fairy shrimp, thereby supporting the hypothesis.

As indicated in previous studies, BTI selectively kills mosquitoes by lysing the epithelial cells of the anterior midgut (Rey, et al., 1998). Since mortality in *B. lindahli* is not significantly affected by BTI, it is suggested that this cell bursting mechanism is not effective on this species of fairy shrimp. Results from Rey *et al.* also show that rapid renewal of epithelial cells in the midgut of *Cladocera ssp.* could also explain the nontoxicity of BTI on fairy shrimp (1998). This may
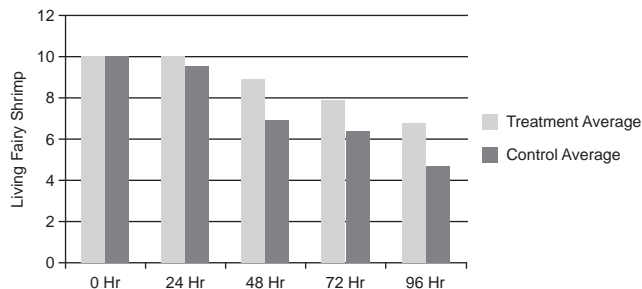
Figure 5

Average number of living fairy shrimp for each day, for a total of 96 hours.
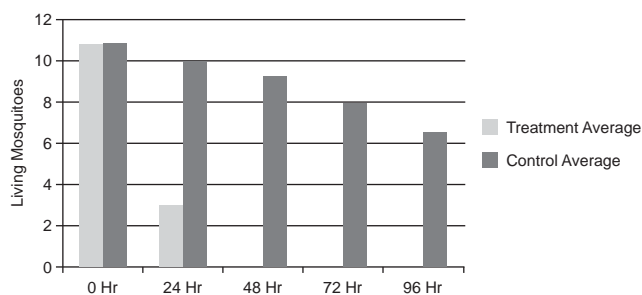
Figure 6

Average number of living mosquitoes for each day, for a total of 96 hours.

explain why fairy shrimp are capable of surviving in the treated water. Bravo *et al.* demonstrated that mutations in receptors for the BTI toxins decrease the affinity for BTI binding, which increases resistance to effects of BTI on other invertebrates (Bravo, et al., 2007). However, *B. lindahli* has not yet been tested and further studies are needed to determine what specific physiological reactions or steps lead to resistance to BTI in the *B. lindahli* midgut.

Brown *et al.* found that mortality rates were not significantly different in *Caradina indistincta*, another family of shrimp, for controls and water treated with BTI (Brown et al., 2000). Similar results were seen in this experiment; in fact, fairy shrimp lived longer in treated waters than in the controls. A likely theory is that competition may produce this unexpected result. As the mosquito larvae die, the fairy shrimp no longer have to compete against them for resources. This idea, however, has not been confirmed and must be further studied.

BTI is a safe, efficient alternative to the more toxic and indiscriminant pesticides used in the past. Although the recommended dosage was tested in this study, the application of BTI can far exceed the suggested concentration. It has been demonstrated to be safe for non-target organisms at several thousand times the recommended dosage and is considered

to be one of the most selective mosquito larvicides (Brown et al., 2000). The opportunistic nature of mosquitoes allows them to invade many different ecosystems and spread infectious diseases to humans. For humans to eradicate these diseases, BTI is the preferred method. As a result, not only can humans protect themselves, but they can also ensure the survival of fairy shrimp and other organisms from the introduction of harsher toxins into ecosystems.

By safely testing the application of BTI in experimental conditions first, damaging sensitive ecosystems like vernal pools can be avoided. Ensuring that these ecosystems remain in balance is crucial because of the highly specialized adaptations seen in fairy shrimp and other organisms endemic to vernal pools. By selectively reducing mosquito populations, humans can maintain ecosystem diversity while protecting themselves from serious diseases. Because *B. lindahli* is an indicator species of vernal pools, its continued presence will suggest that these ecosystems are healthy and sustained year after year. Thus, by saving this unique species, biodiversity can be maintained in aquatic ecosystems.

Author Profiles

Catherine A. Drake
Catherine began working in Dr. Bowler's lab in her third year at UC Irvine, and came to love working in the local freshwater marsh. She has come to appreciate the rarity of the southern California marsh ecosystem, and has been grateful for the opportunity to work toward protecting its unique collection of plants and animals from invading exotic species. Catherine graduated from UC Irvine in 2010 and is planning to attend graduate school, continuing her education in ecology and marine biology.

Owen P. Goldsworthy
Owen considers his time conducting research to be one of the most valuable experiences he gained during his undergraduate years at UC Irvine. He particularly appreciated the opportunity to collect field data in the San Joaquin Freshwater Marsh Reserve, doing important research in such a beautiful natural setting. Having graduated, Owen plans to seek out a position in a research laboratory or hospital and start to put the skills he learned through his research to use.

Paula C. Nuguid
Paula was instantly interested in this project because of the frequent opportunity to visit the vernal pools. She particularly enjoyed being a part of an experiment that is relevant for many people in different disciplines. This experience has increased her awareness of the interconnectedness of things and how small changes can lead to greater outcomes. After graduation, Paula hopes to continue her education and build upon the experience and knowledge she has gained through her research.

Linda J. Patterson
Linda considers the privileges to study within the San Joaquin Marsh Reserve to be one of the best experiences she has have ever had. The most rewarding part was the faith her faculty mentors had in her to make a creative project come to life. She appreciates the opportunity to make a difference at UC Irvine by calling attention to how sensitive the local marsh environments can be. Linda intends to continue her studies in environmental toxicology, eventually entering a specialized field in medicine.

Acknowledgements

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