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SCIENTIFIC NOTE

LACK OF RESISTANCE IN *AEDES VEXANS* FIELD POPULATIONS AFTER 36 YEARS OF *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS* APPLICATIONS IN THE UPPER RHINE VALLEY, GERMANY

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ABSTRACT. *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) has been widely and solely used against floodwater mosquitoes, mostly *Aedes vexans*, for 36 years in the Upper Rhine Valley by the German Mosquito Control Association. During this period, almost 5,000 tons of *Bti* formulations were applied to an area of approximately 400,000 ha. To investigate a possible resistance development after such a long-term and widespread application of *Bti*, the susceptibility of *Ae. vexans* larvae to *Bti* in 3 untreated (Lake Constance) and 6 treated areas on both sides of the Rhine within the Upper Rhine Valley was assessed by bioassays following World Health Organization guidelines. Comparing log-probit analyses, it was shown that neither the median lethal concentration (LC_{50} values) nor slopes of the probit lines of bioassays of the larvae deriving from treated and untreated areas showed significant differences. These results have been confirmed by resistance ratios, which varied from 0.80 to 1.12 in all tests. The results provided the evidence that no restistance in the target species *Ae. vexans* has developed in the areas of the Upper Rhine Valley, despite the large-scale use of *Bti* for 36 years.

KEY WORDS *Aedes vexans*, bioassay, *Bti*, resistance, Upper Rhine valley

In the Upper Rhine Valley, floodwater mosquitoes play an important role as nuisance species and can significantly reduce the quality of life of the residents. The most abundant species is *Aedes vexans* (Meigen), which frequently makes up more than 90% of the mosquito population during the summer (Becker and Ludwig 1983, Becker 1997). In response to this nuisance, 100 communities on both sides of the Rhine River merged their common interest into a united mosquito control program, the German Mosquito Control Association (Kommunale Aktionsgemelnschaft zur Bekämpfung der Stechmückenplage-KABS) in 1976. In an integrated control program, at the beginning a lecithin surface layer (Liparol) was used along with water management. Since 1981 formulations of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) were solely used against floodwater mosquitoes. All *Bti* formulations were sterilized by gamma radiation of 25 kGy and contained no viable spores or cells of *Bti* (Becker 2002). From 1981 to 2016, 4,988 tons of *Bti* formulations (4,911 tons of granules and 77 tons of powder and fluid concentrates) were successfully applied in multiple treatments (average 5) to a total of more than 400,000 ha, resulting in at least 90% reduction of the mosquito populations in a treated area of approximately 60,000 ha year after year.

Usually two thirds of the total area were treated by helicopters. From 1982 to 1997, aerial application was implemented with tailor-made *Bti* sand granules: 50 kg of fire-dried quartz sand (1–2 mm diam) were mixed for 5 min with 1.4 liters of vegetable oil and 1.8 kg of *Bti* powder (VectoBac TP, 5,000 ITU/mg), covering an area of about 2 ha. Overall, 1,100 tons of sand granules were applied over 44,111 ha. In 1998, *Bti* sand granules were replaced by *Bti* ice granules (Becker 2003). The production of ice granules took place by means of fluid nitrogen: 1,000 liters of water were mixed with 40 kg of VectoBac WDG (3,000 ITU/mg) and transformed into icy pearls. From 1998 to 2016, 221,476 ha were treated with 3,811 tons of *Bti* ice granules.

Ground application within the Upper Rhine Valley took place by means of knapsack sprayers. For the treatment of 1 ha, 500 g of VectoBac WDG, or 1 liter of VectoBac 12AS, were mixed with 10 or 9 liters of water (resulting in 10 liters of suspension per knapsack sprayer) and applied by about 300 field workers. Between 1981 and 2016, about 145,687 ha were treated by ground application, using 77.4 tons of powder/fluid formulations. The overall number of rounds of treatments during the 36-year period was 189. Thus 189 populations (generations) of *Ae. vexans* have been subjected to selection pressure of *Bti*. Treatments were usually necessary when egg horizons of the floodwater mosquitoes were flooded (Becker 1989). The horizons corresponded to the middle water level and above of the Rhine River. The number of treatments varied from year to year owing to fluctuations in the Rhine water level. Mass hatching of floodwater mosquitoes peaked between April and September. On average, there were 1 to 11

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floods per year, with an average of 5 floods per summer during the 36 years of *Bti* applications.

One of the main threats for an effective mosquito control is the ability of the target organisms to develop resistance to most control agents used. To address this concern, after 10 years of treatment with *Bti* within the Upper Rhine Valley, the KABS conducted its first investigation in 1991 to detemine if resistance had developed under constant selection pressure of *Bti* treatments (Becker and Ludwig 1993).

This study was based on a comparison of the susceptibiliy of *Ae. vexans* populations obtained from selected untreated areas (Lake Constance) and treated areas (Upper Rhine Valley), which are about 300 km apart (Becker and Ludwig 1993). A similar study was repeated every third year (Ludwig and Becker 1997, Ludwig and Becker 2000, Ludwig and Becker 2005) to monitor mosquito resistance to *Bti*.

To compare the susceptibility of *Ae. vexans* larvae from different field populations, soil samples containing eggs of *Ae. vexans* were collected in the following sites: 1) untreated sites, namely, 3 separate sites on the shore of Lake Constance, and 2) 6 treated areas under certain selection pressure for 36 years of *Bti* applications within the Upper Rhine Valley. Hereby, 3 areas were chosen on the left bank of the Rhine River and 3 sites on the right bank (Fig. 1). Within the larval habitats, 1 m^2 soil samples from the upper layer (approximately 1 cm deep) were removed with a trowel and brought to the laboratory. In total, 9 samples were collected from each of the 3 untreated and 6 treated sites. The samples were kept for 14 days at 25°C to ensure conditioning of the eggs (Becker and Ludwig 1981, Becker 1989). After this period the soil samples were flooded in plastic vessels (40 × 40 × 20 cm) with a water layer up to 20 cm above the soil. The hatched larvae were determined under a stereomicroscope, reared at 25°C, and fed with fish food (Tetramin, Tetra GmbH, Melle, Germany).

All bioassays were conducted with late third instars according to World Health Organization guidelines (WHO 1981), with slight modifications to meet the specific needs of this study. To prepare a stock solution, 50 mg of gamma-irradiated *Bti* (VectoBac WDG, Valent BioSciences, Libertyville, IL) were added to 10 ml of destilled water and homogenized in a mixing machine (IKA Combimag REO) at 700 rpm for 10 min, then homogenized in an ultrasonic bath (Branson Instruments, St. Louis, MO) for 15 min. One milliliter of the homogenized solution was added to 99 ml of destilled water. Depending on the concentration, a range of 15 to 1,500 µl of homogenized, diluted suspension was added to 200 ml plastic cups, which had been previously filled with 148 ml of distilled water. To each cup, 25 larvae of *Ae. vexans* were added in 2 ml of water. Tests were run at 6 different concentrations with controls and replicated 9 times per sampling site.

Larval mortility was evaluated at 24 and 48 h postexposure. Mortality data were corrected, using Abbott's formula (Abbott 1925), if needed. The results were subjected to log-probit analysis (Finney 1971, Raymond 1985), and data means were compared using the Duncan's multiple range test and Student's *t*-test (Köhler et al. 1984).

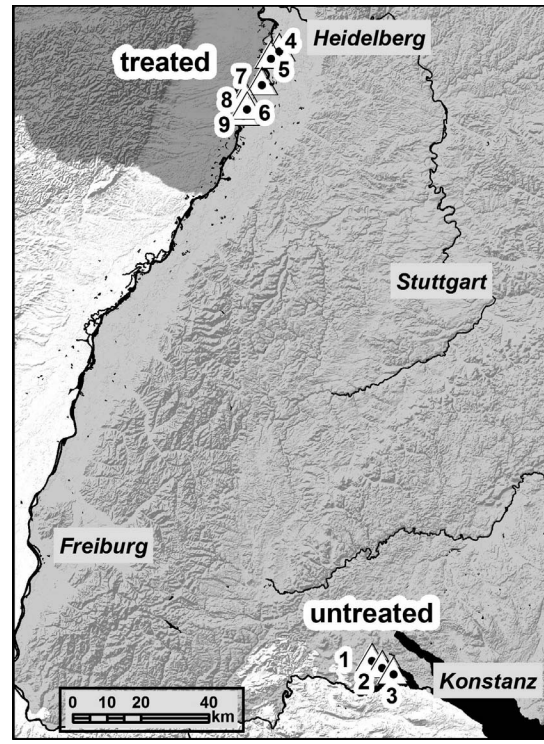

Fig. 1. Sampling sites of soil containing eggs of *Aedes vexans* in *Bti* untreated (1–3) and treated (4–9) areas.

The median lethal concentration (LC_{50} values) and slopes of concentration-response lines conducted with larvae from from 3 indvidual untreated control sites and 6 treated sites showed no significant differences (Table 1). Furthermore, there were no significant differences between average LC_{50} and slope values of untreated sites and treated sites (Table 1), and the average LC_{90} followed the same trend. The resistance ratios (RRs) of the 6 samples from the treated sites as compared with the average susceptibility of the samples from the untreated sites ranged from 0.80 to 1.12 with an average of 0.97, below the cutoff RR of 3, and showed that no resistance developed in the treated sites (Table 1).

The development of resistance is a major problem in controlling insects with chemical and biological insecticides (Mallet 1989, Su 2016). In contrast, the possibility of rapid development of resistance against *Bti* seems to be unlikely because of the complex mode of action of numerous synergistic endotoxins. Nevertheless, a very low level of resistance against *Bti* can be induced in the laboratory when mosquito populations were subjected over a long period of selection pressure (Davidson 1992). For example, Vasquez-

Table 1. The 48-h LC_{50}, slope, and resistance ratios for all groups after 36 years of *Bti* applications ($N = 9$).

Group	LC_{50} (± SD, mg/liters)[1]	Slope[1]	RR[2]
Untreated areas			
Site 1	0.04467 ± 0.00426	2.78	n/a
Site 2	0.03850 ± 0.00857	2.95	n/a
Site 3	0.04328 ± 0.01593	2.42	n/a
Average	0.04147 ± 0.01090	2.71	n/a
Treated areas			
Site 1	0.03331 ± 0.00748	2.54	0.80
Site 2	0.03558 ± 0.00419	2.72	0.86
Site 3	0.04456 ± 0.00335	3.11	1.07
Site 4	0.04177 ± 0.00234	2.78	1.01
Site 5	0.03952 ± 0.00705	2.62	0.88
Site 6	0.04660 ± 0.00194	3.11	1.12
Average	0.04022 ± 0.00678	2.82	0.97

[1] Values under the LC_{50} levels and the slope values are not significantly different among the replicates, averages, or areas ($P \geq 0.05$).
[2] Resistance ratio at LC_{50} = Value of individual treated sites/value of the average untreated sites.

Garcia (1983) treated laboratory populations of *Culex quinquefasciatus* Say with *Bti* at varying levels of selection over 32 generations and found a 5–7-fold decrease in susceptibility. The resistance phenomenon almost completely disappeared after a period of 3 generations without selection pressure. Goldman et al. (1986) found a 2-fold increase in the resisance ratios in only 1 out of 3 populations of *Aedes aegypti* (L.) exposed to *Bti* pressure (LC_{50} values) for 14 generations. Similarly, Gharib and Szalay-Marzso (1986) reported a 1.9-fold increase in LC_{50} values under *Bti* pressure for 25 generations. Georghiou et al. (1983) found an 11-fold decrease of the *Cx. quinquefasciatus* susceptibility to *Bti* after 32 generations, in response to a higher selection pressure (LC_{95}). On the other hand, no or only very low resistance was detected in field population mosquitoes treated with *Bti* (Liu et al. 2004, Akiner et al. 2009, Vasquez et al. 2009, Loke et al. 2010). In contrast to the foregoing reports, Paul et al. (2005) found significant levels of resistance in a field population of *Cx. pipiens* (L.) in an urban area in Syracuse, NY, treated for some years with *Bti* formulations.

The insecticidal effect of *Bti* strains against some members of nematoceran insects, especially mosquitoes and blackfly larvae, emanates from the parasporal body (crystal protein), which contains 4 major peptides of different molecular weights, referred to as Cry4Aa, Cry4Ba, Cry11Aa, and CRY11Aa (Federici et al. 1990, Wirth et al. 2005). A fifth toxin, called the Cyt1Aa protein, is the principal factor for delaying the evolution and expression of resistance to mosquitocidal Cry proteins (Wirth et al. 2005). It is the synergism of the Cry proteins and the CytA proteins that results in the high toxicity against mosquito and black fly larvae. Each single Cry toxin is mosquitocidal, but none is as toxic as the intact combination of all toxins. Selections with single purified toxins or combinations of less toxin in the absence of Cyt1A toxin resulted in the onset of resistance at various places depending on complexity of the combinations (Georghiou and Wirth 1997, Wirth et al. 2005). Similarly, the selection of *Ae. aegypti* with field-persistent *Bti* collected from breeding sites resulted in a moderate level of resistance, as opposed to relatively high levels of resistance to individual Cry toxins. A 3.5-fold tolerance to Cry4Aa and 8-fold resistance to Cry11Aa was detected in one *Ae. sticticus* (Meigen) population (Tetreau et al. 2013).

There may be several reasons why resistance was not found in *Ae. vexans* in spite of 36 years of extensive use to combat floodwater mosquitoes with *Bti*. First is the complex mode of action of all intact toxins. It is assumed that the lethal changes within the cells are produced by the synergstic effects of the 4 different proteins of the parasporal body, particularly the spore-making toxin Cyt1A (Federici et al. 1990). With only protein crystals as in *Btkurstaki* (Tabashnik et al. 1990) or binary toxins in *Lysinibacillus sphaericus* (Sinègre et al. 1994), the chance for a faster development of resistance seems to be much higher (Su 2015). Second, the coevolution between the target insects and the mosquitocidal bacilli for more than 300 million years results in an effective toxin composition. It can be assumed that whenever the insects started to develop resistance against the toxin, the bacilli changed the composition of their toxins to keep the toxicity against the target organims. The ecological advantage of the soil bacterium *Bti*, namely, to kill the target, is that the insect cadaver can serve "as a small fermenter" for the reproduction of the bacillus (Becker et al. 1995). Thus the insecticidal bacteria can better multiply in the soil, which is usually poor in nutrients. Third, variable gene pools exist within target populations. *Aedes vexans* is a strong flyer that facilitates the gene flow between untreated and treated areas, delaying the development of resistance. Maybe untreated areas serve as refugee sites for the conservation of the gene pool. The phased eclosion of *Ae. vexans* produces generations that are not homogenous, which leads to an increase in diversity of the gene pool within the populations (Becker 1989). Last, the rather short exposure period of the toxins and quick kill may also be attributable to lack of resistance development. The confrontation between the toxins and the target organisms takes place only for a short time after field application.

In summary, *Bti* has the unique story of success in combating mosquitoes in Germany and many other countries worldwide. It offers quick kill, high efficacy, and safety to the nontargets, the environment, and the applicators. Most strikingly, no resistance has developed after 36 years of massive application. It is anticipated that *Bti* will continue to play an important role in mosquito control for decades to come.

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