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The bio-larvicide *Bacillus thuringiensis* var. *israelensis* is effective against *Aedes koreicus*, either dissolved in water or delivered through eco-compatible chitosan-based hydrogels

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ABSTRACT

Mosquito control, which is not always easily accomplished, is further complicated by the spread of invasive species. This is the case of *Aedes koreicus*, a mosquito native to East Asia, whose presence has been recorded in several European countries, including Italy. This mosquito found suitable ecological conditions in central Europe in general, and in northern Italy in particular, as shown by the ongoing expansion of its distribution. While basic knowledge on feeding habits of *Ae. koreicus* have already been acquired, information on its vectorial competence is scarce. Therefore, active monitoring on the presence of this mosquito, and the pre-planning of future control actions, are of paramount importance. Currently, there are no specific guidelines for controlling this mosquito, both in its native regions and in invaded countries. Here we present the first study on the efficacy of a bio-insecticide based on *Bacillus thuringiensis* on *Ae. koreicus* larvae, with a comparison with results obtained on the tiger mosquito *Aedes albopictus*. Our results proved that this bioinsecticide is effective on *Ae. koreicus*, both dissolved in water and incorporated into MosChito raft, a hydrogel-based matrix that has recently been developed for the delivery of insecticides to other mosquito species and suitable for safe and eco-compatible applications.

1. Introduction

During the last few years, the invasive mosquito *Aedes koreicus* has considerably expanded its geographical distribution and is now listed among the alien insect species that have invaded Europe (Capelli et al., 2018; Liu et al., 2023). *Aedes koreicus* is native to East Asia; in Europe, this mosquito has so far been recorded in Austria, Belgium, Germany, Hungary, Italy, the Netherlands, Romania, Russia, Slovenia, and Switzerland (Versteirt et al., 2012; Werner et al., 2016; Negri et al., 2021). The expansion of *Ae. koreicus* across Europe might represent an emerging threat to the health of humans and animals, due to the potential role of these mosquitoes as vectors of a variety of pathogens. Indeed, experimental and field studies highlight the possibility that this mosquito might be involved in the transmission of viruses (chikungunya, Zika, and Japanese encephalitis viruses) and filarioid nematodes, such as *Dirofilaria immitis* (Ciocchetta et al., 2018; Ganassi et al., 2022).

In Italy, *Ae. koreicus* has rapidly expanded its distribution, likely from one or a few initial introduction sites (Capelli et al., 2011; Montarsi et al., 2013; Montarsi et al., 2015). It might spread further in the future, in suitable areas (Arnoldi et al., 2022), a pattern different from that in populations of *Ae. koreicus* described in Germany and Belgium, which, apparently, remained restricted to the areas where they have first been recorded (Hohmeister et al., 2021). Resistance of adult *Ae. koreicus* to dry environments, and the adaptation of the larvae to relatively cold temperatures, minimize competition with other species, allowing exploitation of novel breeding niches, possibly with higher fitness in

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some specific conditions, compared to congeneric mosquitoes (Baldacchino et al., 2017; Liu et al., 2023).

Application of control measures is thus necessary to curb the proliferation of *Ae. koreicus* populations, to limit their further expansion, and then to control the risks associated with their potential role as vectors of viruses and filarioid nematodes. Actions to control mosquitoes include the use of larvicides. *Bacillus thuringiensis* var. *israelensis* (*Bti*) is regarded as a highly effective and eco-compatible larvicide and is recommended for the control of *Aedes* spp. mosquitoes in several countries worldwide (Brühl et al., 2020). This biopesticide is active by ingestion, since the bacteria produce proteinaceous toxins that target the midgut epithelium of mosquito larvae (Brühl et al., 2020; Silva-Filha et al., 2021). Prior to field application of this larvicide, it is important to establish baseline information on the susceptibility of the target mosquitoes present in a given area. *Bti* doses for the control of *Aedes albopictus* have already been defined, and protocols and recommendations for practical use are available (Baldacchino et al., 2015). However, the efficacy of *Bti* against *Ae. koreicus* has not yet been investigated. This study was designed to make a first contribution to filling this knowledge gap.

The present study was designed to evaluate the toxicity of *Bti* against *Ae. koreicus*, compared to toxicity in *Ae. albopictus*. This is relevant to an important issue: these mosquito species can exploit the same water pools as breeding sites. For example, should *Ae. koreicus* be less susceptible to *Bti* compared to *Ae. albopictus*, the dosages used to control the latter could favour the evolution of resistance in the former. Another critical aspect in the use of bioinsecticides is the design of strategies that ensure proper stability of the insecticide in the environment and an effective delivery to target insects. Therefore, we also tested the efficacy of *Bti* on *Ae. koreicus*, after its incorporation into a hydrogel matrix, MosChito raft, developed for the delivery of insecticides to mosquito larvae (Piazzoni et al., 2022; Negri et al., 2023; Pitton et al., 2023).

2. Materials and methods

2.1. Mosquito strains

Bioassays were performed using larvae of *Ae. albopictus* Levate strain (Pitton et al., 2023) and *Ae. koreicus* Bergamo strain. Both strains have been recently established (September 2020) from larvae collected during a surveillance programme for invasive mosquito species in the province of Bergamo district (Italy). The colonies were maintained in the insectary under standard rearing conditions (Pitton et al., 2023), larvae were fed on fish food (Tetra-fish, Melle) and adults on sucrose solution (10% w/v in distilled water). Females were fed on animal blood to allow egg development. Eggs, laid on filter paper in Petri dishes, were hatched through a nutrient broth (Pitton et al., 2023).

2.2. Susceptibility of *Ae. koreicus* larvae to *Bti* under laboratory conditions

The susceptibility of *Ae. koreicus* larvae to a *Bti*-based insecticide was investigated under controlled laboratory conditions (24 ± 2 °C), following the WHO protocol (WHO, 2015) and using scaled multiple doses of the commercial VectoBac®12AS (Sumitomo Chemicals Italia SRL, Valent Biosciences, Milano, Italy). Pools of 25 early fourth-instar larvae of *Ae. koreicus* were treated in plastic cups with 100 ml of tap water and different concentrations of VectoBac®12AS (0.1, 0.2, 0.25, 0.3, 0.37, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, and 1.1 mg/l). In each assay, four replicates for each dose were performed; tap water was used as control. The bioassay was repeated three times, analysing the mortality at 24 h and 48 h. The same experiment was performed also on *Ae. albopictus*, following the same protocol, to compare the susceptibility of the two species. Probit analysis to determine LD_{50} and LD_{90} values and 95% fiducial confidence limits were performed with PoloPlus 2.0 software (LeOra Software, Berkeley, CA, USA).

2.3. Susceptibility of *Ae. koreicus* larvae to *Bti* delivered by MosChito raft in semi-field conditions

After determining the susceptibility of *Ae. koreicus* larvae to VectoBac®12AS, larval response to MosChito rafts was tested under semi-field condition. MosChito hydrogel rafts were prepared as previously described in our publications (Piazzoni et al., 2022; Negri et al., 2023; Pitton et al., 2023), producing two types of rafts: a control empty raft, and a *Bti* + Y raft containing cells of *Saccharomyces cerevisiae* (strain SY2080; 10^7 cells/raft) and VectoBac®12AS at a final concentration of 420 μl/ml (Negri et al., 2023; Pitton et al., 2023).

Pools of 50 larvae at different developmental stages (from first- to fourth-instar) were treated into the insect breeders (Bug Dorm provided by NHBS GmbH, Bonn, Germany) containing 200 ml of field-collected water. The experiments were carried out under conditions mimicking a hypothetical larval breeding site, in the inner courtyard of the university (Negri et al., 2023).

Mortality was evaluated every 24 h until all the treated larvae were dead or pupated. Statistical analysis was carried out with GraphPad Prism (GraphPad Software Inc. version 8.0), by the Log-rank (Mantel-Cox) test and between-group comparison, with FDR (false discovery rate)-adjusted *P*-values.

3. Results

3.1. Susceptibility of *Ae. koreicus* and *Ae. albopictus* larvae to *Bti* under laboratory conditions

Bioassays on *Ae. koreicus* larvae were performed to determine susceptibility of this species to the entomopathogen *Bti*. Larvae were exposed to the commercial *Bti*-based product VectoBac®12AS, used for mosquito larvae control. Mortalities of *Ae. koreicus* larvae after 24 h and 48 h of exposure under laboratory conditions are shown in Fig. 1. There was a very slight difference in mortality between 24 h and 48 h post-treatment in terms of dose-response efficacy. A slight increase in response rate was observed after 48 h with the lower doses, denoting greater potency of the insecticide as treatment time increases.

To compare susceptibility of *Ae. koreicus* and *Ae. albopictus* larvae, bioassays were also performed in parallel using larvae from a colony of *Ae. albopictus* established in the same period of *Ae. koreicus* colony. The results obtained demonstrated that *Ae. koreicus* larvae were slightly less susceptible than those of *Ae. albopictus* (Table 1). In particular, the *Bti* lethal doses (LD_{50}, LD_{90}, LD_{99}) at different time points (i.e. 24 h and 48 h), were different between the two species: in general, *Ae. albopictus* larvae were killed with a lower dose of *Bti*, compared with the larvae of *Ae. koreicus*.

Thus, the present data confirm that the commercial *Bti*-based product VectoBac®12AS is effective against *Ae. koreicus* in laboratory conditions and that the effective dose is quite similar, compared to the ones active on *Ae. albopictus* and *Culex pipiens* (*s.l.*) (Negri et al., 2023; Pitton et al., 2023).

3.2. Delivery and efficacy of *Bti* against *Ae. koreicus* larvae through MosChito rafts

The survival rate of *Ae. koreicus* larvae treated with the *Bti* delivery device MosChito raft in semi-field conditions is shown in Fig. 2. The pools of larvae at different developmental stages demonstrated a high susceptibility to *Bti* + Y MosChito raft, exceeding 50% mortality already on Day 1 of treatment. From Day 1 to Day 2, the survival range fell from 15% to 7%, reaching 100% mortality on Day 7 post-treatment. On the other hand, mortality of larvae exposed to the empty MosChito raft did not exceed 20%, until the end of the experiment (88% survival rate was observed on Day 7).

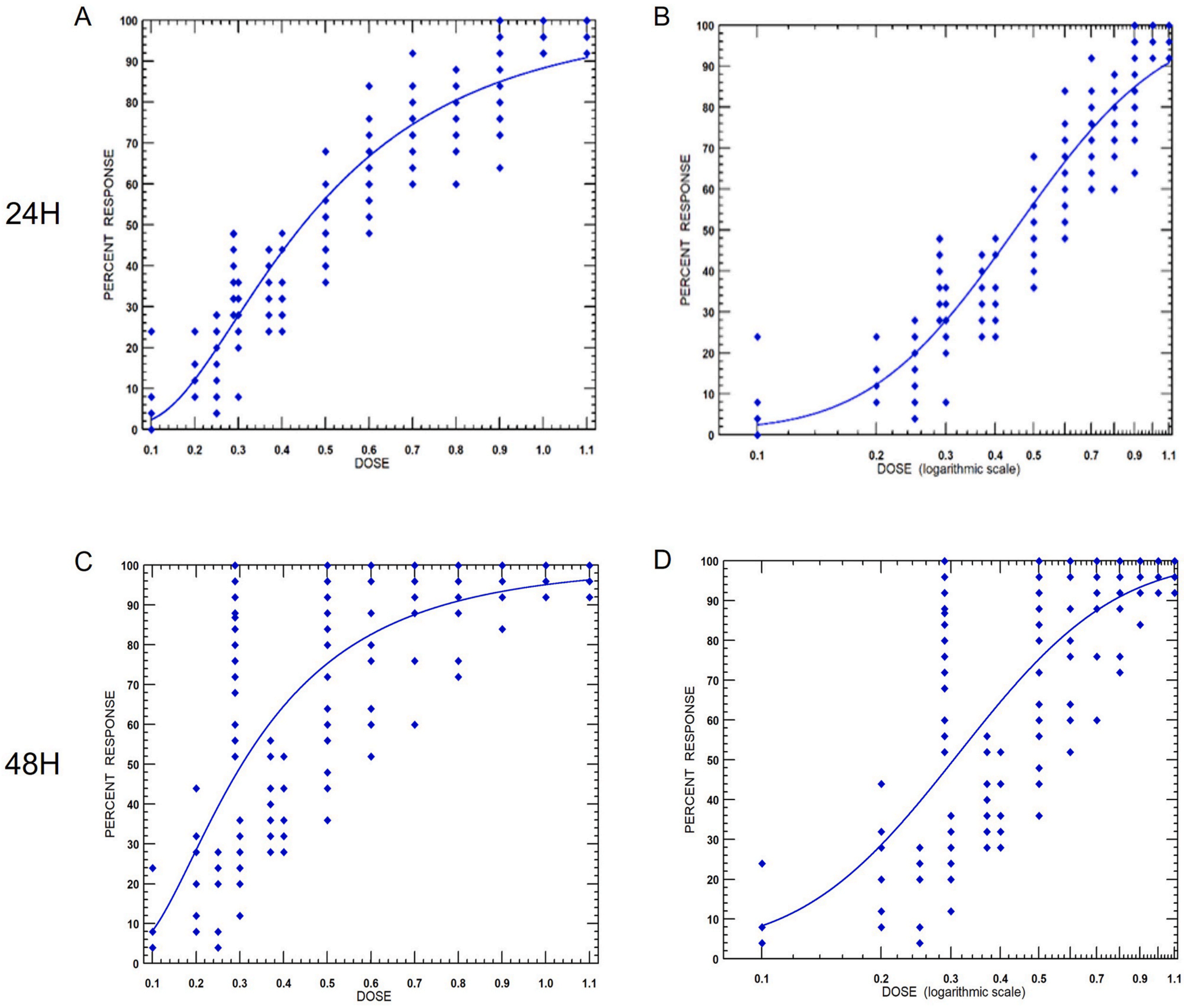

Fig. 1. Mortality of *Ae. koreicus* larvae in bioassays after 24 h and 48 h, under laboratory conditions. Fourth-instar larvae of *Ae. koreicus* were exposed to different doses of the *Bti*-based product VectoBac®12AS (from 0.1 to 1.1 mg/l) and survival was recorded after 24 h (**A** and **B**) and 48 h (**C** and **D**). The dose-response curves in linear scale are shown in **A** and **C**, while the sigmoidal dose-response curves in logarithmic scale are shown in **B** and **D** ($n = 3$ independent experiments).

Table 1
Bti lethal doses (LD) for *Ae. koreicus* and *Ae. albopictus* larvae in bioassays after 24 h and 48 h, under laboratory conditions.

Lethal doses	Time points	Lethal doses (95% CI) in mg/l	
		Ae. koreicus	*Ae. albopictus*
LD_{50}	24 h	0.451 (0.434–0.469)	0.382 (0.364–0.400)
	48 h	0.312 (0.283–0.339)	0.320 (0.293–0.345)
LD_{90}	24 h	1.068 (0.999–1.153)	0.774 (0.714–0.854)
	48 h	0.777 (0.700–0.885)	0.629 (0.571–0.715)
LD_{99}	24 h	2.154 (1.918–2.467)	1.786 (1.483–2.281)
	48 h	1.631 (1.352–2.094)	1.090 (0.921–1.382)

4. Discussion

In this study, the efficacy of a *Bti*-based insecticide (i.e. VectoBac®12AS) was evaluated, on different larval stages of *Ae. koreicus*, as it represents a major tool for the biological control of several mosquito species in Europe and in other regions worldwide (Brühl et al., 2020). In the first part of the study, we determined the timing of larval death after *Bti* exposure, as well as the LD_{50}. These were similar to those recorded for *Ae. albopictus*. However, *Ae. koreicus* resulted slightly less susceptible to *Bti* compared with *Ae. albopictus.* These results are on the one hand encouraging, in that they highlight the possibility of using the same commercial product for the control of both mosquito species. However, based on our results, the dosages currently used for the control of *Ae. albopictus* could possibly be less effective on *Ae. koreicus* larvae. Therefore, further studies are required, in order to optimize *Bti*-based interventions, when planned with the objective of controlling both *Ae. albopictus* and *Ae. koreicus*. On the other hand, methods for the delivery of *Bti* to *Aedes* spp. larvae could possibly allow to increase the efficacy of this bioinsecticide.

Bti is an environmentally friendly larvicide, suitable for applications in water pools possibly exploited by different species of mosquitoes. We emphasize that the ecological habits of *Ae. koreicus* and *Ae. albopictus* are very similar: they are container-breeding mosquitoes, able to lay eggs in different types of natural or anthropogenic water pools (Montarsi et al., 2013, 2022; Medlock et al., 2015). At the same time, the adaptation of *Ae. koreicus* to low temperatures, resulting in earlier spring hatching of larvae and a postponed winter diapause phase, makes it an efficient invader, which presents potentially limited competition towards *Ae. albopictus*, thanks to an alternation in the use of breeding sites (Baldacchino et al., 2017).

Bti acts in a targeted but effective manner: in tricky and sensitive

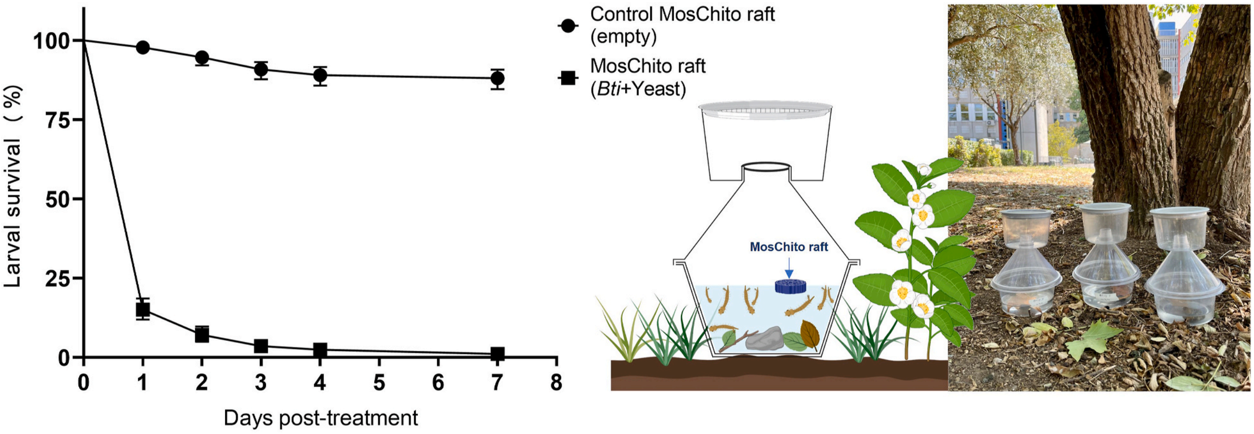

Fig. 2. Mortality of *Ae. koreicus* larvae exposed to MosChito rafts in semi-field conditions. Pools of different instar larvae of *Ae. koreicus* were exposed to control MosChito raft (empty matrix), or *Bti* + Y MosChito raft (matrix with *Bti* and yeast), and mortality was recorded every 24 h. The results are represented as the mean ± standard error ($n = 3$ independent experiments) ($P < 0.0001$).

environments, such as urban and peri-urban settings, *Bti* maintains good balance between instances related with human health (i.e. the control of mosquito vectors) and the preservation of insect biodiversity (Schäfer and Lundström, 2014; van den Berg et al., 2015; Brühl et al., 2020). These characteristics of *Bti* could be enhanced through the use of eco-compatible matrices, suitable for the preservation and delivery of this bioinsecticide. In this context, MosChito raft has been shown to further the larvicidal efficacy *Bti*, on both laboratory- and wild-collected strains of *Ae. albopictus* and *Culex pipiens* (*s.l.*), ensuring long-lasting action and facilitating the delivery of the bioinsecticide to mosquito larvae (Piazzoni et al., 2022; Negri et al., 2023; Pitton et al., 2023). A key feature of MosChito raft is its attractiveness for the larvae of *Ae. albopictus* (Pitton et al., 2023). In addition, *Aedes* larvae exhibit a pronounced "gatherer" and "shredder" behaviour on detritus (Wallace, 2008). Indeed, when exposed to MosChito raft, mosquito larvae actively erode the hydrogel matrix, with a consequent ingestion of the incorporated VectoBac®12AS bioinsecticide (Pitton et al., 2023). In the present study, the new semi-field experiments on *Ae. koreicus* showed that MosChito raft is effective also on this species, with a good delivery of *Bti* to the larvae, as proved by mortality data.

Comparing the present results in semi-field conditions on *Ae. koreicus* with those obtained in our previous work on *Ae. albopictus* (Pitton et al., 2023), from the first 24 h of treatment it was evident that the larvicidal effect of MosChito rafts is more rapid on *Ae. koreicus* (survival of 15% *vs* 43% respectively, Day 1). This result is quite interesting considering the lower susceptibility of *Ae. koreicus* larvae to *Bti* dissolved in water (see *Section 3.1*). Therefore, the problems that could derive form the lesser susceptibility of larvae from *Ae. koreicus* to water-dissolved *Bti* could be overcome after incorporating the bioinsecticide into MosChito rafts. The faster action of MosChito rafts + *Bti* on *Ae. koreicus*, compared to *Ae. albopictus*, might possibly derive from the greater size of the larvae of the former (unpublished observation). This greater size could possibly imply a greater capability of the larvae from *Ae. koreicus* to shred the hydrogel, with a consequent increased ingestion the *Bti.* Whereas the slight delay in reaching 100% mortality for *Ae. koreicus*, compared to *Ae. albopictus*, leading to a one- or two-day bioassay time extension, could be the consequence of the slower development of the early larval stages, that are possibly not very effective in the shredding of the hydrogel. Indeed, in our experience in the maintenance of laboratory colonies of *Ae. koreicus* and *Ae. albopictus*, the former species reaches the fourth-instar larval stage in approximately 10 days, while the time required for the latter is approximately 6–8 days (personal observations).

5. Conclusions

To the best of our knowledge, our results provide the first evidence for the efficacy of *Bti* against *Ae. koreicus*, either dissolved in water or administered through the MosChito raft device. *Bti*, and MosChito raft + *Bti*, could thus contribute to integrated vector management plans for the control of mosquitoes (Bellini et al., 2014, 2020), against both native and alien species, an issue of paramount and increasing importance in urban and peri-urban settings.

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Ethical approval

Not applicable.

CRediT authorship contribution statement

Agata Negri: Conceptualization, Methodology, Investigation, Writing – original draft. **Giulia Pezzali:** Methodology, Formal analysis. **Simone Pitton:** Methodology, Formal analysis. **Marco Piazzoni:** Methodology, Investigation, Project administration. **Laura Soresinetti:** Methodology, Writing – review & editing. **Giovanni Naro:** Conceptualization, Formal analysis, Writing – review & editing. **Paolo Gabrieli:** Validation, Writing – review & editing. **Giorgia Bettoni:** Writing – review & editing. **Claudio Bandi:** Supervision, Funding acquisition, Writing – review & editing. **Silvia Caccia:** Supervision, Writing – review & editing. **Sara Epis:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Data availability

The data supporting the conclusions of this article are included within the article and its Supplementary file S1.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2024.100197.

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