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Effects of Selection to Diflubenzuron and *Bacillus Thuringiensis* Var. *Israelensis* on the Overwintering Successes of *Aedes albopictus* (Diptera: Culicidae)

Charalampos S. Ioannou [1,2], Christos Hadjichristodoulou [1], Varvara A. Mouchtouri [1] and Nikos T. Papadopoulos [2,*]

[1] Laboratory of Hygiene & Epidemiology, Faculty of Medicine, School of Health Science, University of Thessaly, 41222 Larissa, Greece; ioannoubabis@yahoo.com (C.S.I.); xhatzi@med.uth.gr (C.H.); mouchtourib@uth.gr (V.A.M.)
[2] Laboratory of Entomology & Agricultural Zoology, Department of Agriculture Crop Production and Rural Environment, School of Agricultural Sciences, University of Thessaly, 38446 Volos, Greece
* Correspondence: nikopap@uth.gr

Simple Summary: *Aedes albopictus* is an invasive mosquito species well established in many parts of Europe and posing high risks of autochthonous transmission of chikungunya and dengue viruses. The high dependency on commonly used larvicides such as Diflubenzuron (DFB) and *Bacillus thuringiensis* var. *israelensis* (*Bti*) to control its populations pose major concerns for resistance development. Although studies addressing the potential resistance development and evolution of *Ae. albopictus* in the wild against these two important larvicides are essential for planning control programs, no such data are available. Here, by imposing an increasing selective pressure for nine successive generations against both DFB and *Bti* on a recently laboratory established *Ae. albopictus* population we determined the induced resistance levels and the overwintering success of the selected populations relative to control (colonies that received no selection). Our findings revealed a substantial and a moderate increase on the resistance levels following selection with DFB and *Bti* respectively. Moreover, no significant differences were observed regarding the overwintering successes between the two selected populations and the control, highlighting the ability of the resistant individuals to persist in the wild from year to year.

Abstract: *Aedes albopictus* is an invasive mosquito species responsible for local transmission of chikungunya and dengue viruses in Europe. In the absence of available treatments, insecticides-based control is one of the most important viable strategies to prevent emerging problems. Diflubenzuron (DFB) and *Bacillus thuringiensis* var. *israelensis* (*Bti*) are among the most commonly used larvicides for *Ae. albopictus* control which pose major concerns for potential resistance development. Studies on the resistance emergence of *Ae. albopictus* to both DFB and *Bti* and its persistence in the wild are essential for the efficient and sustainable planning of the control programs. In this concept, larvae from a recently laboratory established population were subjected to increasing selective pressure for nine successive generations against both DFB and *Bti*. At the end of this process, the resistance levels and the overwintering success of the selected populations relative to control (colonies that received no selection) were determined. Results revealed a substantial and a moderate increase on the resistance levels following selection with DFB and *Bti* respectively. Interestingly, the selection process to both larvicides had no apparent impacts on the overwintering capability relative to control suggesting the successful persistence of the resistant individuals in the wild from year to year.

Keywords: Asian tiger mosquito; eggs diapause; insecticides efficacy; mosquito control

1. Introduction

Aedes albopictus Skuse (Diptera: Culicidae) also known as the Asian tiger mosquito is classified as one of the 100 most invasive species in the world [1]. During the last 50-60 years, largely facilitated by human activities, it has extended its geographic distribution from its native range in tropical and subtropical Southeast Asia to all over the world [2]. Successful colonization of the novel habitats in the Northern Hemisphere has been attributed to the remarkable ecological and physiological plasticity of this species [3]. In temperate regions, *Ae. albopictus* overwinters at the egg stage containing pharate 1st instar larvae in facultative diapause that occurs when maternal females experience short day photoperiod as pupae and adults [4]. In Europe, *Ae. albopictus* was recorded for the first time in Albania in 1979 [5], however awareness only arisen in the early 90s after the discovery of established populations in Italy [6]. To date, it displays a wide-range distribution in both the Mediterranean basin and central Europe having established in at least 19 countries [7]. Due to its aggressive biting behavior, *Ae. albopictus* causes important nuisance problems that negatively affect the welfare levels while they may also act as an important discouraging factor for the tourism industry [8]. Moreover, as a competent vector of more than 32 arboviruses, including chikungunya and dengue, *Ae. albopictus* poses a major threat for public health [9]. In Europe, its presence has been associated with autochthonous transmission of dengue in Croatia (2010), France (2010, 2013-2015) and Spain (2014-2016) and of chikungunya in Italy (2007 and 2017) and southeastern France (2010 and 2014) [10]. Furthermore, as a consequence of its highly opportunistic feeding behavior on different animal species, *Ae. albopictus* has the potential to act as a bridge-vector of zoonotic pathogens from animal-to-animal and from animal-to-human [2].

In the absence of commercially available vaccines or treatments, *Ae. albopictus*-borne diseases such as dengue and chikungunya may be prevented mainly through vector control [11]. In recent years, new control approaches have been proposed such as the Sterile Insect Technique (SIT), the Release of Insects carrying a Dominant Lethal (RIDL), the release of *Wolbachia*-infected mosquitoes and pyriproxyfen autodissemination i.e. a self-delivery technique that manipulates mosquitoes' behavior to carry and disseminate the killing agent (pyriproxyfen) to potential breeding sites [12]. Despite the encouraging results, certain limitations prevent the wide application of these approaches in the field. Among others, the main limitations in the area-wide application of SIT against mosquitoes are the high production and transportation costs of sterile males required to achieve successful suppression of the wild populations [13]. On the other hand, the implementation of both RIDL and *Wolbachia*-based strategies are under strict prohibition in many countries including the European Union due to environmental/ecological concerns, whereas the efficacy of pyriproxyfen autodissemination is highly depend on the availability of breeding sites in a given area [12]. Therefore, insecticide applications still remain the principal tool for tackling *Ae. albopictus* related problems.

In general, insecticide interventions prioritize larval over adult control, because of the higher expected impact and the lower environmental costs. Currently in Europe, larval control relies almost exclusively on the Insect Growth Regulator (IGR) Diflubenzuron (DFB) and the microbial control agent *Bacillus thuringiensis* var. *israelensis* (*Bti*) [14]. DFB is a member of the Benzoylurea insecticide family that inhibits the chitin biosynthesis process interrupting the insect's normal growth [15]. During sporulation *Bti* produces a mixture of Cry (Cry11Aa, Cry 4Ba, Cry 4Aa and Cry10Aa) and Cyt (Cyt1Aa and Cyt2Ba) toxins [16]. The Cry toxins bind to specific midgut membrane receptors disrupting its integrity, while the Cyt toxins appear to act synergistically with the Cry toxins increasing their capacity to bind on the available target sites (receptors) [17, 18]. The synergistic interactions between the Cyt and the Cry toxins have attracted particular attention due to the drastic enhancement of their larvicidal activity against mosquitoes [19]. Moreover, the synergism between the Cyt1Aa and the Cry toxins is considered the key factor for the low potential of resistance development following extensive selection with *Bti* [16, 20].

The high dependency on both DFB and *Bti* for the suppression of mosquito populations pose major concerns for resistance development. While a recent study suggest their suitability for the effectiveness control of *Ae. albopictus* [21], findings regarding other species does not fully confirm this point of view. In particular, striking DFB resistance levels associated with specific mutations have already been detected in *Cx. pipiens* natural populations with a focal distribution in areas with intense use of this insecticide [22, 23]. On the other hand, there is only a single record of high (33-fold) *Bti* resistance levels in wild mosquito populations with a background of previous exposure to support for potential resistance development [24]. Despite the prominence of these findings, no efforts have been made so far to explore the potential resistance development of *Ae. albopictus* to DFB and *Bti*.

In general, insecticide resistance development is often associated with significant fitness costs as a result of trade-offs associated with the function of the resistance mechanisms [25]. Moreover, adverse abiotic conditions may largely shape the expression of fitness costs related to insecticides selection in mosquitoes [26]. In this regard, winter in temperate regions represents a very challenging season for the survival of mosquitoes and therefore potential insecticide resistance associated fitness costs may reduce their overwintering capability. This is of high importance as it can determine the survival of resistant mosquitoes from year to year affecting both the evolution and establishment of acquired resistance in the wild. While the effects of DFB and *Bti* selection on the overwintering success of the medically important mosquito species *Cx. pipiens* have recently been elucidated [26], no such information is available for *Ae. albopictus* given its different winter biology. Considering the above mentioned knowledge gaps regarding the potential performance of *Ae. albopictus* to DFB and *Bti* exposure, the aim of the present study was to explore how selection to both larvicides affect the resistance development as well as the overwintering success of this species.

2. Materials and Methods

2.1 Mosquitoes used and rearing methods

An *Ae. albopictus* population was established during early September to mid-October of 2017 from approximately 2000 eggs that were collected using a network of 20 ovitraps set up at the vicinity of Volos and Larisa city, Thessaly county, Greece. The ovitraps were consisted of glossy black, cylindrical (15 cm high and 11 cm diameter) plastic pots filled with 800 mL tap water up to an overflow hole. Ten wooden strips (tongue depressors of 15 × 1.8 cm) with one side properly scratched by a serrated knife, were attached with paper clips vertically in the inner of each pot serving as oviposition substrates. Strips from each oviptap were collected every 8-10 days, placed in plastic sealing bags and transferred to the laboratory. Colonization took place within the insectary facilities of the laboratory of Entomology and Agricultural Zoology at the University of Thessaly. The insectary walk-in chamber was maintained at 25 ± 1 °C, 65 ± 5% relative humidity and a photoperiod of L14 : D10 with a simulated dusk and dawn for 45 min. Larvae were reared in 42 × 30 × 10 cm^3 white plastic containers in 3 L of bottled table water (Table Water, Epirotic Bottling Industry S.A. Ioannina, Greece), fed a total amount of 2 g of ground cat food (Friskies Adult, Purina, Italy) and held at a density of approximately 1000 individuals per container. Adults were kept in 32 × 32 × 32 cm^3 screened cages at a density of 600-700 individuals and fed with 10 % sugar solution that was renewed every week. Mosquitoes were offered an arm feeding by a volunteer (C. Ioannou) until all responding females (usually 80-90 % of total) in each cage completed a full blood meal. Females had access to oviposit on moist filter papers submerged in 500 mL of tap water inside black, plastic cylindrical containers. Mosquitos were reared for 3 generations to establish a uniform genetic background before the initiation of the experiments.

2.2 Larval bioassays

Following the standard WHO guidelines for laboratory studies of mosquito larvicides [27] the susceptibility of the established population was evaluated against analytical standard DFB (Purity ≥ 99.8 %, Pestanal®, Sigma-Aldrich, Taufkirchen, Germany) and formulated *Bti* (Vectobac® 12AS, 11.61 % w/w Bti serotype H-14, strain AM65-52, 1200 ITU/mg, Valent BioSciences Corporation, Libertyville, IL, USA). Stock solutions were prepared in 99.5 % acetone for DFB and distilled water for *Bti* and stored at -22 °C until use for up to two weeks. For DFB, six concentrations ranging from 0.001 to 0.005 mg/L and for *Bti* five doses ranging from 0.02 to 0.04 mg/l were used yielding larval control between 10 and 95%. Six replicates were performed per concentration and an equal number of controls, each involving twenty five 3rd and early 4th instar larvae for DFB and *Bti*, respectively. Bioassays were run three times on different days using new batches of larvae and larvicide solutions. Larval mortality for *Bti* and adult emergence inhibition for DFB were recorded according to WHO recommended exposure times [27]. For each mosquito population, dose mortality responses were used to calculate IE_{50}, IE_{80} and IE_{90} values (IE: Adult emergence inhibition) for DFB and LC_{50}, LC_{80} and LC_{90} values (LC: Lethal concentration) for *Bti* using Probit Analysis [28].

2. 3 Larval selection

Larvae were selected for six successive generations to fixed concentrations of DFB and *Bti* corresponding to IE_{80} and LC_{80} respectively. At this point, the induced susceptibility levels were recalculated and the selection process was continued for three additional generations by applying the new fixed doses corresponding to IE_{80} and LC_{80} respectively. During the selection process, eight groups of ≈1000 larvae were placed into rearing containers (see above) provided with 3L of table water and the fixed dose of each larvicide. Selection against DFB involved 3rd instar larvae, while 1.2 g of cat food was added in the containers to allow development (pupation). Selection against *Bti* involved the exposure of early 4th instar larvae to fixed doses for 24 h without any accesses to food. Surviving larvae were placed at a maximum density of ≈1000 individuals into rearing containers with 3L of clean table water and offered 1 g of food to complete development. Resulting pupae from both DFB & *Bti* selection process, were transferred daily into cages and reared following the standard procedures described above. Additionally, two larvae groups (≈1000 individuals each) were maintained under identical conditions but in the absence of DFB & *Bti* exposure serving as controls. There was at no point exchange of individuals among treatments and controls during the above mentioned procedures. After the completion of the final (ninth) selection, new dose-response bioassays were performed to establish the induced resistance levels against both DFB & *Bti* following the standard WHO guidelines described before. Moreover, the winter survival of diapausing eggs originating from both the selected populations and the control was evaluated.

2.4 Winter survival

To induce *Ae. albopictus* females from each experimental population to lay diapausing eggs, both immature and adult stages were reared in an environmental chamber set at 20 °C, 8L:16D, and 70% RH [29]. Seven to ten days after adult emergence, females were offered a blood meal (arm feeding). Soon after, 200 blood fed females from each population were allocated in two rearing cages (100/cage) and allowed to lay their eggs for 15 days in two ovitraps (one/cage), each containing 15 wooden strips. Ovitraps were then removed from the cages and held for 7 days inside the environmental chamber to permit embryonation. Then, eggs were cold acclimated inside an incubator at 10 ± 1°C, RH 75 ± 5% and constant darkness for a week to enhance their cold hardiness [4]. Before exposed outdoors, each wooden strip was pictured under a binocular stereoscope (ZEISS, SteREO, Discovery.V12, Oberkochen, Germany) equipped with a digital camera (ZEISS, AxioCam, ERc 5s) to facilitate eggs counting. Then, strips were clipped to the inner edge of ovitrap pots with a single drainage hole placed 4.5 cm from the bottom. This configuration allowed ambient rain water, to remain and moisten the wooden strips inside the pots but not flood the eggs. Two such pots were considered for each experimental population hosting a total of 30 wooden strips (15 each). Pots were covered, including the drainage hole, with mesh screen (mesh size 1.0 mm) and fitted randomly side by side into a black, plastic, mesh box (mesh size 1 cm). To prevent access of macroscopic organisms, a second box was fastened on the top serving as a cover. Pots were positioned in the corner of a concrete wall at the University facilities, well protected from both direct sun light and strong winds. Eggs exposure took place on November 12, 2019. An outdoor data logger (HOBO Pro v2, Onset Computer Corporation, Bourne, MA, USA) set to receive 4 recordings of both temperature and RH per 24h was attached at the bottom plastic box that hosted the pots. Based on empirical evidences suggesting the first appearance of *Ae. albopictus* adults in the area at the beginning of May overwintered egg strips were collected on April 10, 2020 and transferred to the laboratory. Strips were again pictured under the stereoscope and intact eggs were counted. To stimulate larval hatching, each egg strip was submerged for a week in a white, plastic container with 300 mL stale tap water provided with 0.05 g of cat food. After that, egg strips were removed from the water and left to dry for 72 hours before a subsequent flooding was performed for another week. This procedure was repeated four times, until no hatching was observed, while the remaining unhatched eggs were considered dead. For each experimental population, survival of overwintered eggs under the ambient natural conditions was determined.

2.4 Statistical Analysis

Dose-response larval bioassays were subjected to Probit Analysis by transforming dose to log. For the two selected populations and the control, the numbers of intact eggs in wooden strips before and after outdoor exposure and the respective proportions of hatched larvae were determined. Data were analyzed using One-way analysis of variance after appropriate transformations for normality and homoscedasticity when necessary followed by Tukey's HSD post hoc to separate means. Data analysis was performed using IBM SPSS 25 (IBM Corp., Armonk, NY).

3. Results

Dose-response larval bioassays results are given in Table 1 & 2 for DFB and *Bti* respectively. The selection process with DFB for six successive generations by applying the EI_{80} corresponding dose, as determined for the established population, resulted in 3.6 and 4.4 Resistance Ratio (RR) values relative to control as far as EI_{50} and EI_{90} are concerned. Selection for three additional generations with the new established EI_{80} corresponding dose, as determined at the end of the previous selection process (F6 generation), nearly doubled these values (Table 1). On the other hand, the selection processes against *Bti* had minor effects on the induced susceptibility levels (Table 2). Selection against both DFD and *Bti* reduced female fecundity relative to control, however no significant differences were observed, suggesting a rather moderate fitness cost (Table 3). Recovery rates of diapaused eggs after outdoor exposure reached more than 95 % for all tested populations (Table 3). Ambient temperature and relative humidity conditions during eggs' overwintering are depicted in Figure 1. Temperatures ranged between 3.8–19.3 °C with a mean value of 10.9 °C indicating a rather mild cold period. Selection to *Bti* significantly increased the survival rates of overwintered eggs relative to control and the *DFB* selected population (Table 3). Moreover, the hatching rates of viable eggs (i.e those yielded larvae) following the first immersion in the water was significantly higher in both selected populations relative to control suggesting a better synchronization on adults' appearance.

Table1. Effective doses of diflubenzuron (DFB) against *Ae. albopictus* before and after the selection process for six and nine successive generations respectively.

Population	N*	EI$_{50}$ (95% CL)[a]	RR$_{50}$	EI$_{90}$ (95% CL)[a]	RR$_{90}$	Slope	X^2 (df)
Pre-selection							
Colony	3150	0.0017 (0.0014-0.0019)	-	0.0039 (0.0036-0.0041)	-	3.56	119.24 (105)
Post-selection							
Control	2700	0.0022 (0.0016-0.0026)	-	0.0041 (0.0037-0.0045)	-	4.68	156.25[b] (87)
F6 DFB	2700	0.0080 (0.0070-0.0089)	3.6	0.0182 (0.0170-0.0196)	4.4	3.58	62.23 (87)
Control	2700	0.0022 (0.0019-0.0025)	-	0.0040 (0.0038-0.0043)	-	4.86	99.94 (87)
F9 DFB	2700	0.0188 (0.0174-0.0200)	8.5	0.0301 (0.0288-0.0319)	7.5	6.28	24.73 (87)

[*] Number of larvae tested. [a] EI values are expressed in milligrams per liter, and they are considered significantly different when 95 % of confidence limits (CL) fail to overlap.[b] Since goodness-of-fit test is significant ($p < 0.05$), a heterogeneity factor was used in the calculation of confidence limits (CL).

Table2. Effective doses of *Bti* against *Ae. albopictus* before and after the selection process for six and nine successive generations respectively.

Population	N*	LC$_{50}$ (95% CL)[a]	RR$_{50}$	LC$_{90}$ (95% CL)[a]	RR$_{90}$	Slope	X^2 (df)
Pre-selection							
Colony	2700	0.027 (0.024-0.034)	-	0.051 (0.047-0.056)	-	4.59	94.44 (87)
Post-selection							
Control	2700	0.032 (0.030-0.034)	-	0.046 (0.044-0.050)	-	8.08	132.02[b] (87)
F6 *Bti*	2700	0.041 (0.031-0.048)	1.3	0.067 (0.062-0.071)	1.5	6.04	59.86 (87)
Control	2700	0.030 (0.028-0.032)	-	0.045 (0.043-0.047)	-	7.70	58.39 (87)
F9 *Bti*	2700	0.047 (0.036-0.055)	1.6	0.083 (0.076-0.088)	1.8	5.20	80.62 (87)

[*] Number of larvae tested. [a] LC values are expressed in milligrams per liter, and they are considered significantly different when 95 % of confidence limits (CL) fail to overlap.[b] Since goodness-of-fit test is significant ($p < 0.05$), a heterogeneity factor was used in the calculation of confidence limits (CL).

Table 3. Female fecundity and winter larvae survival parameters of Ae. albopictus diapausing eggs originated from populations that either were selected against diflubenzuron (DFB) and Bti for nine successive generations or not (control). For each population, 200 blood fed females were allowed to lay their eggs in 30 wooden strips.

Population	Mean ± SE Eggs/Wooden Strip Before Outdoor Exposure	Eggs/Wooden Strip After Outdoor Exposure	Hatching Rates of Recovered Eggs %	Hatching Rates of Viable Eggs % During the First Water Immersion
Control	304.47 ± 23.70 [a]	294.10 ± 23.14 [a]	59.98 ± 1.31 [b]	85.50 ± 1.80 [b]
F9 DFB	279.63 ± 17.36 [a]	273.57 ± 17.20 [a]	60.15 ± 1.35 [b]	91.71 ± 0.87 [a]
F9 *Bti*	266.47 ± 25.07 [a]	257.60 ± 24.73 [a]	66.17 ± 1.96 [a]	93.35 ± 1.32 [a]
F	0.75	0.73	5.05	8.96
df	2, 87	2, 87	2, 87	2, 87
P	0.476	0.485	0.008	<0.0001

Means in the same column followed by the same letter indicate no significant differences (Tukey's HSD test, $p > 0.05$).

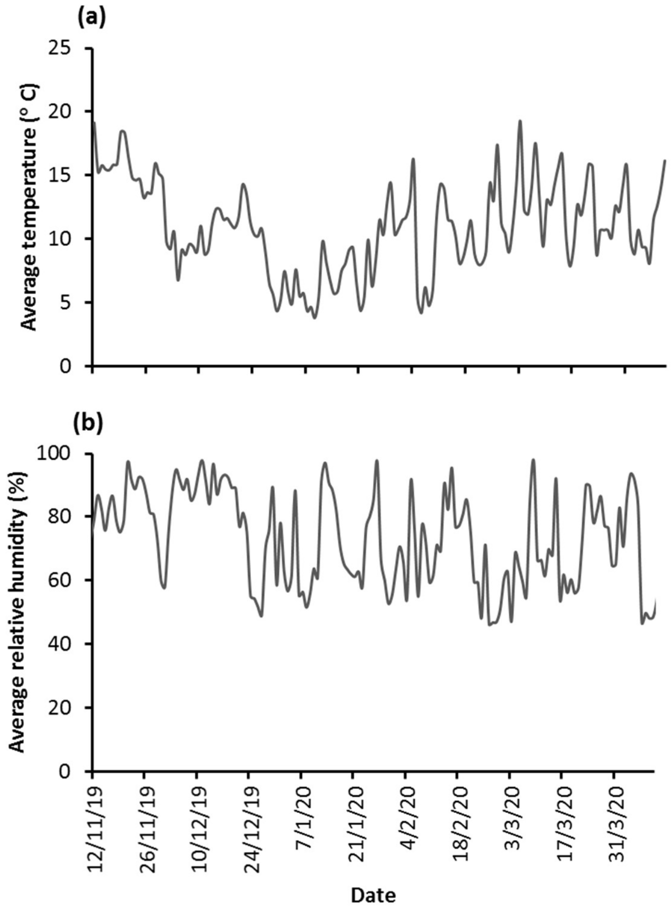

Figure 1. Temperature (a) and humidity (b) profiles at the study site during winter 2019/2020.

4. Discussion

Despite the recent advances on new management approaches, the control of *Ae. albopictus* still relies to a great extent on the utilization of insecticides raising concerns over resistance development. Understanding both the evolution and stability of acquired resistance in the wild is crucial for the sustainability of management programs. In this concept, the current study represents a comprehensive investigation on the resistance development and establishment against two widely used larvicides. By applying a progressively increasing selective pressure with DFB and *Bti* to *Ae. albopictus*, over a relative small number of successive generations, we found a considerable and a minor decrease on the susceptibility levels respectively. Interestingly, selection to both larvicides had no apparent fitness costs in terms of female fecundity and the winter survival of diapausing eggs relative to control (non-selected population). These findings provide important insights into the early phase of resistance development of *Ae. albopictus* against these two important larvicides, but mostly on its prevalence in the wild from year to year.

The EI_{50} value estimated in the current study for the established population is remarkably lower than the recommended WHO dosage of DFB in potable water containers (0.25 mg/L) [30] as well as the emergence inhibition dose (EI_{50} of 0.376 mg/L) reported for field collected *Ae. albopictus* populations from southern Switzerland [11]. In support to our findings, a recent survey revealed 100% mortality of *Ae. albopictus* populations collected from different regions of Greece at DFB doses below 0.02 mg/L suggesting also high susceptibility [21]. On the other hand, the estimated LC_{50} value for *Bti* was 4.8 to 7.5-fold lower compared to the respective values recorded for other Greek populations of this species according to the previously mentioned study. However, such variations are not uncommon as an earlier study in the island of Cyprus revealed an over 10-fold range in *Bti* LC_{50} values for wild collected mosquito populations of *Cx. pipiens* [31]. Interestingly, the LC_{50} value determined in our study (0.027 mg/L) is less than reported for laboratory strains of *Ae. albopictus* suggesting high susceptibility to *Bti*. Indicatively, Li et al. 2018 [32] and Su et al. 2019 [33] report LC_{50} values 0.036 mg/L and 0.044mg/L respectively for control, susceptible strains using a *Bti* formulation with a higher potency than in our case (7000 vs 1200 ITU/mg).

Despite the high susceptibility to DFB of the established population, selection process resulted in the development of considerable resistance levels. During the first six successive generations of selection to the fixed dose corresponding to EI_{80}, only a moderate increment on the $RR_{50\ \&\ 90}$ values was observed. This is in line with the results obtained by Belinato & Valle [34] who applied exactly the same selection protocol on *Aedes aegypti*. However, selection for three additional generations with the new established EI_{80} corresponding dose, nearly doubled these values, indicating that the resistance development and evolution to DFB is highly facilitated by the imposition of increasing selective pressure. Thus, it appears that under certain conditions, even highly susceptible populations of *Ae. albopictus* as in our case, are capable of developing substantial resistance levels. While, recent evidences suggest the high suitability of DFB for the Asian tiger mosquito control [21], this is partially true for other medically important mosquito species. In particular, striking DFB resistance levels associated with specific mutations have already been detected in *Cx. pipiens* natural populations [22, 35]. Moreover, further studies reveled a high focal distribution of resistant *Cx. pipiens* mosquitoes, which was attributed to the high selective pressure imposed by both agricultural and mosquito control applications with DFB in the specific areas [23]. Therefore, in the light of our findings, the possibility of focal DFB resistance patterns, as in the case of *Cx. pipiens*, cannot be excluded for *Ae. albopictus*, emphasizing the need for systematic resistance monitoring.

On the other hand, selection to *Bti* had a negligible impact on the resistance levels of *Ae. albopictus* established population. With the exception of a single case in *Cx. pipiens* from New York, USA [24] there are no consistent recordings of mosquito resistance to *Bti* [36]. The more or less unchanged mosquitoes' susceptibility to *Bti* after extensive periods of applications in the wild or intensive laboratory selection have been primarily attributed to the synergistic action between the Cry and the Cyt toxins, as previously mentioned. Conversely, selection using single *Bti* toxins can rapidly lead to considerable resistance levels [37-39]. Another aspect that appears to facilitate the lack of *Bti* resistance in mosquitoes is the fact that in the absence of selection pressure, any acquired resistance disappears almost completely within 3-5 generations [40,41]. The high fitness costs following extensive selection to this microbial agent have been proposed as the most convincing explanation.

Although, selection against both DFD and *Bti* reduced female fecundity compared to control, no significant differences were observed, suggesting a moderate fitness cost. In contrast to our findings, *Cx. pipiens* selection to *Bti* for 20 generations resulted in 44.8% decrease of female fecundity [40] while Belinato & Valle [34] by applying the same experimental protocol as we, found that DFB selection against *Ae. aegypti* for 6 generations also conferred a significant reduction on this parameter. However, it should be stressed that in these studies, tested females were previously exposed at larval stage to *Bti* and DFB respectively. Therefore, these results indicate primarily delayed effects following larvae exposure to sub-lethal doses of *Bti* and DFB rather than actual fitness costs. On the other hand, the females in our study that laid the diapausing eggs had no prior contact as immatures to both larvicides and thus we are confident that their oviposition output reflects the net costs induced as a result of the imposed selective pressure.

Survival of overwintering *Ae. albopictus* eggs under natural conditions is highly depend on the exposure methods. In this regard, our exposure configuration resulted in a 60% on average hatching rate of the control population eggs, which is almost identical with the highest rate determined in a recent study that assessed different overwintering exposure protocols [42]. Selection to DFB had no apparent effect on the hatching rates of the overwintered eggs relative to control suggesting no differences in their winter physiology. But most importantly, it means that the progenies of the DFB resistant individuals have the same survival prospects as the susceptible ones, providing the base for the building of higher resistance levels in the next year. In this concept, the appearance of resistance without evidence of a trade-off in overwintering survival may pose a major challenge to future management of *Ae. albopictus* with DFB. Interestingly, while selection against *Bti* had a minor impact on the susceptibility levels, it conferred a moderate, though significant advantage regarding the overwintering success of the tested population relative to control. To the best of our knowledge this is the first report of a potential advantage associated with *Bti* selection in mosquitoes. Similarly to our findings, a positive association between selection to *Bacillus thuringiensis* subsp. *kurstaki* and the overwintering survival of the agricultural pest *Trichoplusia ni* (Lepidoptera: Noctuidae) has been recorded [43]. Authors attributed this phenomenon to possible pleiotropic effects according to which an allele responsible for resistance is positively influencing one of the components related with increased winter survival. However, this justification is not valid in our case as there are no consistent recordings of mosquito resistance to *Bti* as mentioned above and therefore no such alleles have been described for any mosquito species so far. As emerged from the current study, selection to *Bti* resulted to a substantial reduction of female fecundity relative to the other two tested populations (control and DFB selected). Therefore, it is possible that a greater maternal investment in terms of eggs nutrition occurred in this case providing the background for a better overwintering capability.

In the present study, the vast majority of eggs from all tested populations hatched after the 1st inundation followed by subsequent minimal hatchings. This is in line with an earlier study where overwintered eggs went into a mass 1st hatching followed by several additional small ones [42]. *Ae. albopictus* as well as other species of the genus *Aedes*, has a distinctive hatching pattern known as installment hatching. According to this, some eggs may hatch readily following inundation, while others may remain dormant for varying periods despite being submerged [44]. Selection to both DFB and *Bti* resulted to a higher synchronization of larvae hatching during the first inundation relative to control. This is probably because the selection process in both cases enabled the prevalence of individuals with this attribute. While the biological significance of this finding remain largely unknown, at least theoretically it could promote the co-occurrence of the selected individuals during spring facilitating their interbreeding and therefore the establishment of resistant populations in the wild.

5. Conclusions

By using an *Ae. albopictus* population with high, inherent susceptibility to both DFB and *Bti* we showed that exposure to progressively increasing selective pressure resulted in a considerable and a moderate increase of the resistance levels respectively. Moreover, the selection process with both larvicides revealed no significant trade-offs on females' fecundity as well as on the overwintering success of their descendants. These findings provide important insights regarding the resistance development of *Ae. albopictus* against these two widely used larvicides but mostly on its prevalence in the wild form year to year. Based on this, systematic monitoring for potential resistance development is imperative in order to maintain the current effectiveness of these two larvicides against *Ae. albopictus* populations and especially of DFB.

Supplementary Materials: Not applicable

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