Article

Effects of Altosid XR briquets as sustained-release formulations on *Culex pipiens* and honey bees

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Abstract
Mosquitos are dangerous disease vectors to humans. There are many methods to control mosquitos, and one of these methods depends on adding insect growth regulators (IGRs) in pond water to impair the development of immature stages. On the other side, honey bees may collect contaminated water with IGRs to dilute stored honey inside hives for feeding. The interaction between IGRs used to control mosquito and honey bees, as a non-target organism, has gained little attention. In this study, the efficacy period of a single application of Altosid XR (methoprene as an active ingredient) on mosquito, *Culex pipiens*, reared in pond water from two different sources, and its effects on honey bee, *Apis mellifera*, larvae and adults were studied. The results showed that the Altosid as sustained–release formulations had continuous effectiveness with 90-100% inhibition of adult formation for 43 and 45 days post-treatment for mosquito reared in pond water from the two sources. The source of pond water showed no high impact on the efficacy period of Altosid, especially the period with the highest effectiveness. Significantly higher numbers of treated honey bee larvae with Altosid were removed by worker bees than the control group. The survival of adult bees fed on syrup prepared using water collected after two and three weeks post-treatment with Altosid impacted negatively than the control group. Single application with Altosid can be considered as a suitable treatment for mosquitos up to 45 days, meanwhile as a potential hazard to bee colonies for immature and mature stages.

Keywords methoprene; adults; larvae; Altosid XR; IGRs; survival.

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1 Introduction

Mosquitos are a great challenge to humans because they can host and transmit many dangerous diseases including malaria, dengue virus, West Nile virus and Zika virus (e.g. Ito et al., 2002; Joshi et al., 2002; Hayes et al., 2005; Kilpatrick et al., 2008). Hence, the mosquito control efforts are very essential to prevent the outbreak of such diseases. The immature stages of mosquitos live in water while adults are not. Thus, the control methods of immature stages depend on killing them in the water canals using biological enemies including fish (Louca et al., 2009), predators (Zuharah and Lester, 2010) and larval odonates (Saha et al., 2012), bacteria (Regis et al., 2000), or adding some materials. These materials include the use of insect growth regulators (IGRs) (Fournet et al., 1993; Silva and Mendes, 2007; Kamal and Khater, 2010; Msangi et al., 2011; Suman et al., 2013). IGRs can reduce mosquito populations by preventing the development of immature stages. One of these IGRs is Altosid which is a sustained–release formulation and can interrupt the normal development of mosquito larvae, increasing larval and pupal periods, and inhibiting emergence of adults (Farghal and Temerak, 1981). Methoprene is the active ingredient in Altosid and has efficacy against mosquito (Baruah, Das 1996). The efficacy period of this IGR is expected to be changed over time after a single application, and may be vary according to pond water source. Thus, efficacy period, and potential impacts of pond water source on Altosid worth investigations.

One the other side, honey bees, *Apis mellifera*, are very valuable social insects to humans. They contribute in the pollination of many fruit and field crops (e.g. Morse, Calderone, 2000; Kamel et al., 2013). Also, beekeeping represents the main or supplementary source of income to many families (Chazovachii et al., 2013; Qaiser et al., 2013; Al-Ghamdi et al., 2016). Previous studies have shown that chemicals used in the agricultural environment can impact honey bees negatively (Abou-Shaara and Abuzeid, 2018; Zhang et al., 2011; Zhang, 2018). These chemicals include different types of pesticides, herbicides, and few studies have been done on materials used in mosquito control (e.g Caron, 1979; Zhong et al., 2004; Qualls et al., 2010). Honey bees tend to gather water from many resources for regulating relative humidity in colonies (Human et al., 2006; Abou-Shaara et al., 2017) or to dilute stored honey for feeding, and may collect water from unexpected sources including salty water or from wet wooden parts (Abou-Shaara, 2012). The bees may collect water contaminated with IGRs used in mosquito control to dilute honey for feeding, hence exposing immature and mature stages to IGRs. In fact, IGRs can impact negatively immature stages and may be hazardous to honey bee adults (Tasei, 2001). Also, side effects of Altosid have been found against non-target organisms (Miura and Takahashi, 1974; Norland and Mulla, 1975; Lawrenz, 1984). Moreover, methoprene can alter foraging behavior of honey bee as it can increase the number of early foragers (Huang et al., 2016). Thus, studying the potential effects of Altosid on honey bees is required.

In this study, the efficacy period of Altosid against mosquito, *Culex pipiens*, reared in pond water from two different sources was studied. Also, the effects of Altosid on larvae and adults of honey bee, as a non-target organism, were investigated.

2 Materials and Methods

2.1 Efficacy period of Altosid against mosquito

Altosid XR briquets (Zoecon Corporation, Dallas, Texas, U.S.A), contain 1.8% methoprene as an active ingredient and 98.2% inert ingredient were used in this study. Experiments were carried out in suitable glass jars containing 15 liters of pond water taken from two suitable mosquito breeding sites, the first site was from Kafr Eldawar district while the second one from Abo Homos district, and both districts are within El-Behera governorate (Coordinates: 30.61°N 30.43°E) at North Egypt. Each glass jar received one Altosid briquet (46.4 gm) and 4 plastic pots, and in each pot twenty five 2nd instar larvae of *Culex pipiens* were placed with a total of 100 larvae per each test period (Fig. 1). The test period represents the total period from adding the larvae until all larvae died or pupated while the live pupae were transferred to untreated water in clean glass beakers for emergence. New 100 larvae were added per each test period, this procedure was continued sequentially until the effects of Altosid briquets reaches allow level (less than 50% inhibition of adult formation). In parallel with treatment groups, another control groups using pond water without treatments from the two districts were tested following the same procedure of treatments. Larval mortality after 24 h and at the end of each test period, percentage of pupation, percentages of emerged and inhibited adults were recorded and compared between test periods and between the two sources of pond water (i.e. districts) as well as between treatments and control groups.

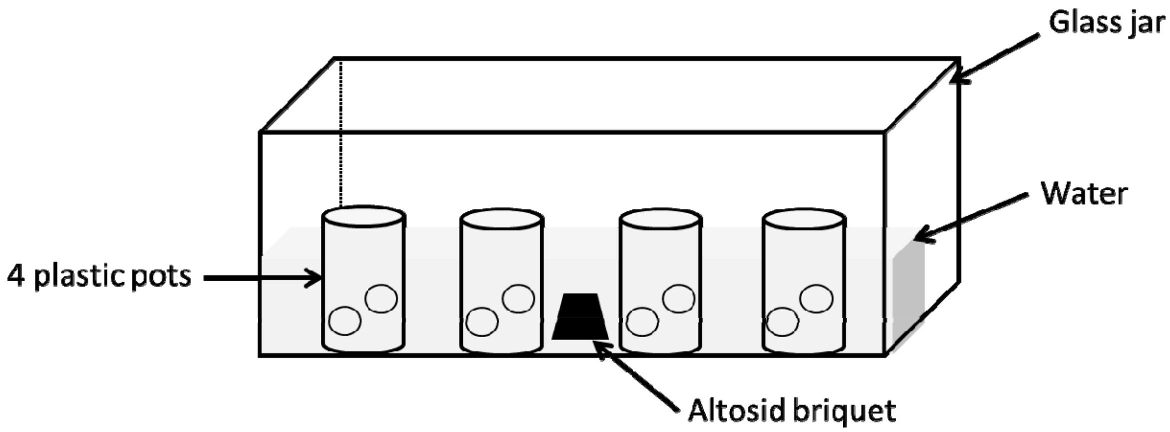

Fig. 1 Illustration of the experimental jars. The glass jars (100×40×30 cm) contain 15 liters of pond water, one Altosid briquet (46.4 gm), and 4 white plastic pots (10 cm long × 13 cm diameter) and in each pot 25 larvae of C. pipiens (a total of 100 larvae). Each plastic pot has four openings (2.5–5 cm diameter) covered by muslin cloth to allow water circulation.

2.2 Effects of Altosid on honey bees

2.2.1 Preparing treatment materials

One Altosid briquet (46.4 gm) was placed in 15 liters of clean water. After one, two, and three weeks, water was collected and used directly to treat larvae, and to feed adults after mixing with sugar syrup. Egyptian Carniolan honey bee colonies from an apiary at Damanhour city, Egypt were used in the experiments.

2.2.2 Immature stages

The older larvae of honey bees at age 3 days and more receive honey and pollen in their feeding, hence the diluted honey with contaminated water with Altosid can reach to them. Also, it is anticipated that very few amounts of Altosid can reach to the larvae. Thus, areas containing from 60 to 73 larvae at age about 3 days were marked on wax combs. Then, the larvae were sprayed with the treatments using 0.25 ml per area. Three colonies were used, and four areas were marked in each colony (three areas for the treatments and one area for the control). The three treatments were; water collected after one week (A1W), after two weeks (A2W), and after three weeks (A3W) post-treatment with Altosid, beside larvae treated only with water as the control group. After 4 days, the numbers of sealed cells (i.e. pupae) were counted as an indicator to the successful pupation. Subsequently, the sealed cells were inspected regularly every 3 days and the numbers of hatched adults were counted. The percentages of pupation were calculated from the counted numbers (= number of pupae/number of larvae X 100), and compared between groups.

2.2.3 Survival of workers

The collected water, after one (A1W), two (A2W), and three weeks (A3W) post-treatment with Altosid was mixed with sugar to make 50% syrup (1 sugar:1 water). These treatments were then tested beside sugar syrup 50% prepared only using clean water as a control group. Therefore, 4 groups were tested in this experiment (group 1: A1W, group 2: A2W, group 3: A3W, and group 4: sugar syrup as control). Concerning honey bees, forager bee workers were collected from the lateral combs of bee colonies (age about ≥21 days). The bees were collected in plastic jars with perforated covers using forceps. In each jar 20 bees were placed, per each group 3 jars were used with a total of 60 bees, and then the bees were fed on A1W, A2W, A3W or the syrup. The survival of bees was recorded daily and up to 10 days, and then compared between groups.

2.3 Statistical analysis

For mosquito experiments, treatments, test periods and districts were considered as the independent factors while the recorded parameters; larval mortality after 24h and at the end of test period, percentage of pupation, percentages of emerged and inhibited adults were considered as the dependent factors. The test periods and districts were compared using analysis of variance (ANOVA), followed by Turkey test. For honey bee larvae, treatments with Altosid were considered as the independent factors while the number of treated larvae, number of pupae, and percentage of pupation were considered as the dependent factors and compared between treatments using ANOVA, followed by Turkey test. The survival analysis of bee workers over the 10 days was done using Kaplan-Meier test and the significant variations between the test groups were detected using the Log Rank (Mantel-Cox test). The statistical analysis at a significant level of $P \leq 0.05$ was performed using SPSS v. 16 (SPSS for Windows, Chicago, USA, 2007).

3 Results

3.1 Efficacy period of Altosid against mosquito

Results of treatment with Altosid XR briquets as sustained-release formulations against larvae of *C. pipiens* are recorded in Table 1 for Kafr Eldawar district. The effectiveness of Altosid decreased over time until no larvae died after 24h with high emergence percentages of adults from 69 to 85% after 73 days from placing the Altosid in the water. The highest efficacy (from 92 to 100 inhabitation percentages of adults) of Altosid was recorded during the first 3 tests with a total period of 45 days. These three tests differed significantly ($P<0.05$) than the other tests in larval mortality at the end of test period, pupation percentage, and percentage of emerged and inhabited adults as shown in Table 1. Based on overall means, the treated water with Altosid caused significant ($P<0.05$) increase in larval mortality and percentage of inhibited adults, and decrease in percentages of pupation and emerged adults than the pond water without treatment (Fig. 2).

Table 1 Means±S.E. of larval mortality, pupation percentages, emergence percentages, and inhibition percentages of mosquitos exposed to Altosid XR over 8 tests and reared in pond water taken from Kafr Eldawar district (100 2nd instar larvae of *C. pipiens* per each test). Means followed by different letter within the same column are significantly different.

Tests	Test period (days)	Larval mortality (%)		Pupae produced (%)	Adults	
		After 24h	At the end of test period		Emergence (%)	Inhibition (%)
1	15	4.00±0.00a	55.00±5.00a	45.00±5.00c	0.00±0.00f	100.00±0.00a
2	14	3.00±1.00ab	60.00±3.65a	40.00±3.65c	4.00±1.63e	96.00±1.63b
3	16	4.00±0.00a	53.00±1.91a	47.00±1.91c	8.00±1.63e	92.00±1.63b
4	13	2.00±1.15ab	39.00±1.91b	61.00±1.91b	43.00±3.41d	57.00±3.41c
5	15	2.00±1.15ab	14.00±1.15c	86.00±1.15a	58.00±2.00c	42.00±2.00cd
6	14	0.00±0.00b	11.00±2.51c	89.00±2.51a	69.00±1.91bc	31.00±1.91de
7	13	0.00±0.00b	8.00±1.63c	92.00±1.63a	80.00±1.63ab	20.00±1.63ef
8	14	0.00±0.00b	8.00±0.00c	92.00±0.00a	85.00±1.00a	15.00±1.00f

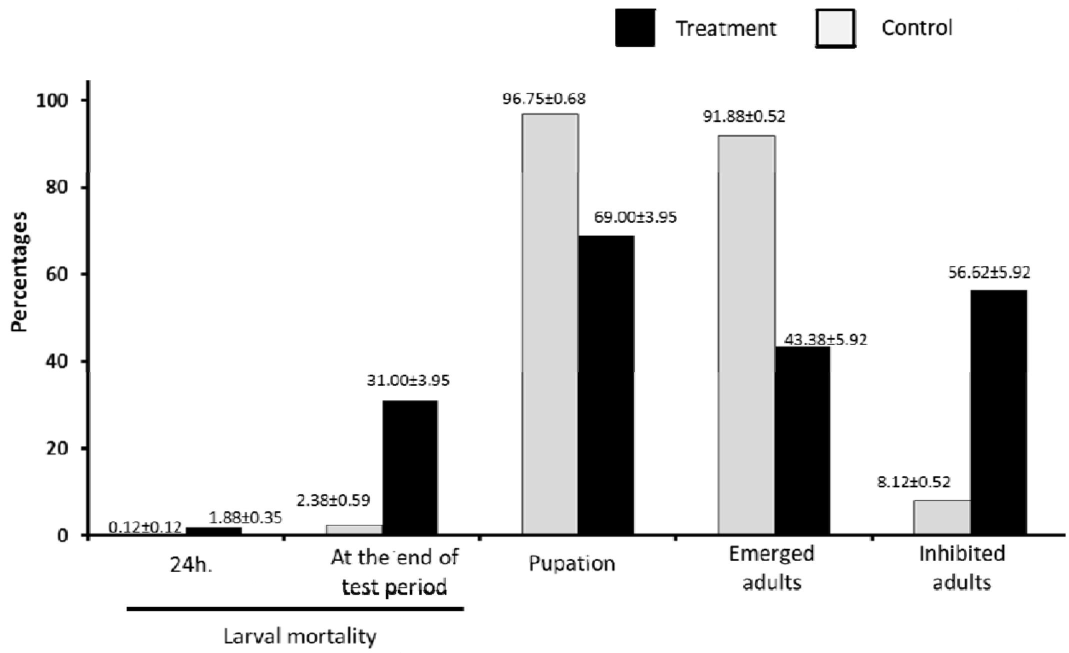

Fig. 2 Overall percentages (Means±S.E.) of larval mortality after 24h and at the end of test period, pupation, emerged and inhibited adults for mosquito *C. pipiens* reared in pond water taken from Kafr Eldawar district without treatment (control) and with Altosid (treatment).

Water collected form Abo Homos district and treated with Altosid showed the ability to increase larval mortality (from 2 to 9% after 24 h) and decrease the emergence rate of adults (from 0 to 9%) during the first 43 days post-treatment (Table2). This ability decreased over time and especially after test 5, as from 59 to 91% of adults were able to emerge. The first 4 tests with high efficacy differed significantly (P<0.05) than the other tests in measured parameters (Table 2). The larval mortality and percentage of inhibited adults increased significantly (P<0.05) while percentages of pupation and emerged adults decreased significantly in treated water with Altosid than the pond water without treatment based on overall means (Fig. 3).

Table 2 Means±S.E. of larval mortality, pupation percentages, emergence percentages, and inhibition percentages of mosquitos exposed to Altosid XR over 8 tests and reared in pond water taken from Abo Homos district (100 2^{nd} instar larvae of *C. pipiens* per each test). Means followed by different letter within the same column are significantly different.

Tests	Test period (days)	Larval mortality (%)		Pupae produced (%)	Adults	
		After 24h	At the end of test period		Emergence (%)	Inhibition (%)
1	9	6.00±1.15a	70.00±2.00a	30.00±2.00d	0.00±0.00d	100.00±0.00a
2	10	3.00±1.00b	65.00±1.91a	35.00±1.91d	0.00±0.00d	100.00±0.00a
3	11	4.00±0.00ab	55.00±1.91b	45.00±1.91c	2.00±1.15d	98.00±1.15a
4	13	2.00±1.15bc	39.00±1.91c	61.00±1.91b	9.00±1.91c	91.00±1.91b
5	15	0.00±0.00c	14.00±2.58d	86.00±2.58a	14.00±3.82c	86.00±3.83b
6	14	0.00±0.00c	11.00±1.00d	89.00±1.00a	59.00±1.91b	41.00±1.91c
7	13	0.00±0.00c	8.00±1.63d	92.00±1.63a	83.00±1.00a	17.00±1.00d
8	14	0.00±0.00c	8.00±0.00d	92.00±0.00a	91.00±1.00a	9.00±1.00d

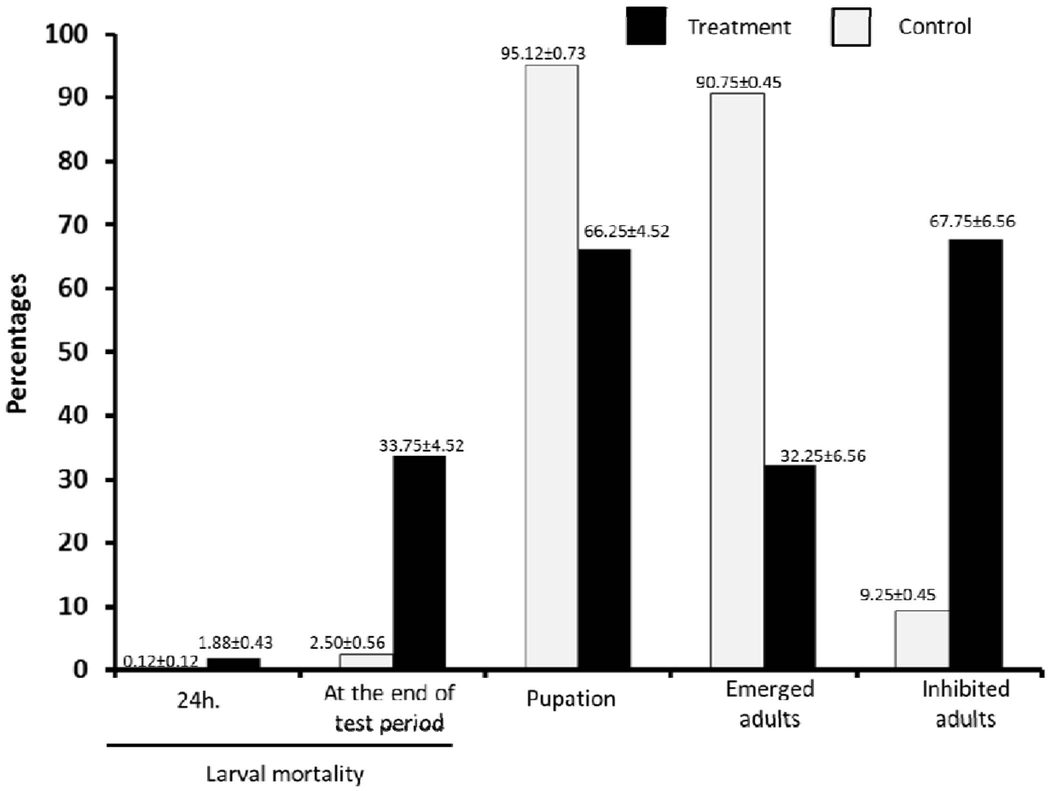

Fig. 3 Overall percentages (Means±S.E.) of larval mortality after 24h and at the end of test period, pupation, emerged and inhibited adults for mosquito *C. pipiens* reared in pond water taken from Abo Homos district without treatment (control) and with Altosid (treatment).

The adult formation using pond water without treatments ranged from 88 to 96% with mean 91.88±0.52% and 90.75±0.45% for Kafr Eldawar and Abo Homos, respectively without significant difference between them. The total period of tests for Altosid placed in pond water collected from Kafr Eldawar and Abo Homos was 114 and 99 days, with mean 14.25±017 and 12.38±0.35 days, respectively. Significant differences ($P<0.05$) were detected between the mean of test periods of the two districts. The efficacy period with inhibition percentage of adult formation above 90% was 45 (three tests) and 43 days (four tests), for the two districts, respectively. The overall means of recorded parameters of larvae, pupae and adults over the 8 tests for the two districts are shown in Fig. 4. No significant differences ($P>0.05$) were detected between the recorded parameters of larvae reared in treated water from the two districts according to ANOVA over the 8 tests. The comparison between the first four tests of the two districts is shown in Fig. 5. Significant differences were found between the two districts in percentages of larval mortality at the end of test period and pupation percentages for test 1 while in the percentages of inhibited adults for the other three tests.

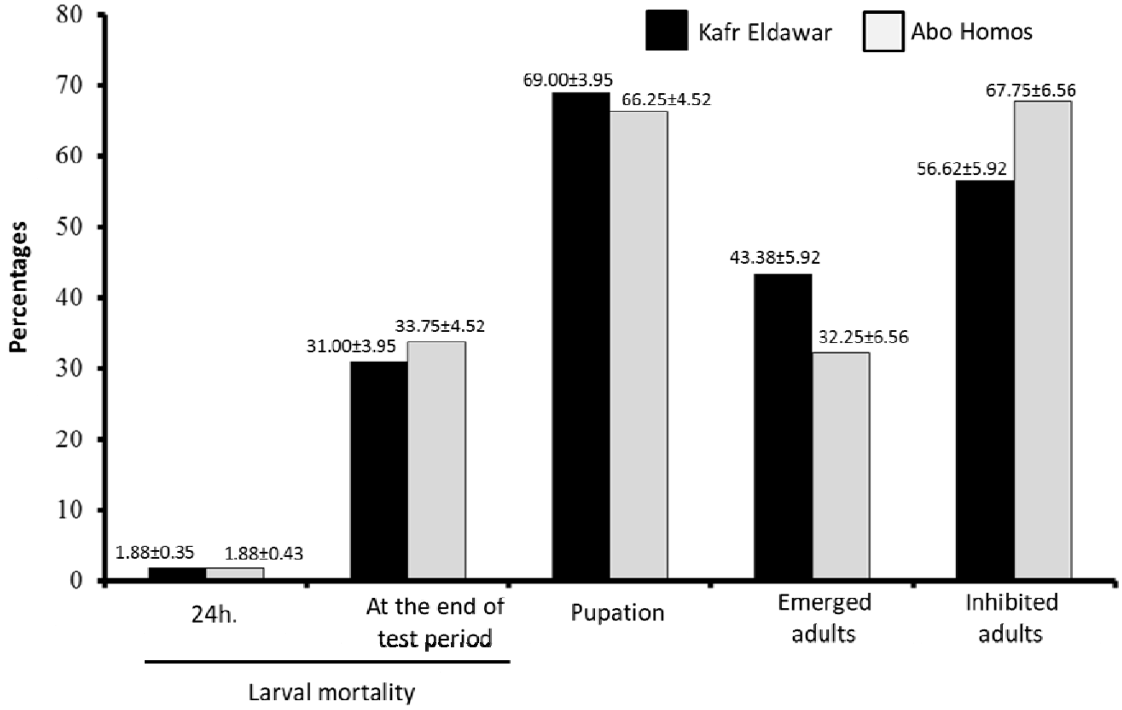

Fig. 4 Overall percentages (Means±S.E.) of larval mortality after 24h and at the end of test period, pupation, emerged and inhibited adults for mosquito *C. pipiens* reared in treated pond water with Altosid taken from Kafr Eldawar and Abo Homos districts.

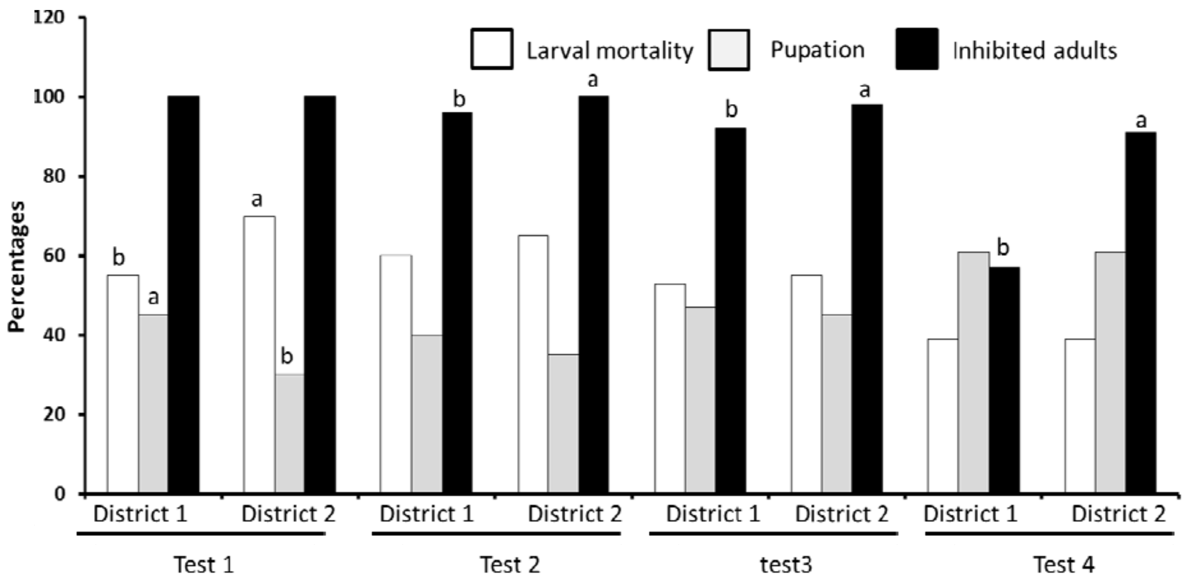

Fig. 5 Mean of larval mortality percentages at the end of test period, pupation, and inhibited adults for mosquito *C. pipiens* reared in treated pond water with Altosid taken from Kafr Eldawar (district 1) and Abo Homos (district 2) for the first 4 tests. The letters a and b denote the significant differences according to ANOVA.

3.2 Effects of Altosid on honey bees

3.2.1 Immature stages

All pupae were able to hatch successfully for all the groups, as no dead or incomplete adults were observed during the regular inspection, hence results of larvae and pupae only are presented in Table 3. The differences between number of treated larvae were not significant (ANOVA; df=3, F=3.331, P=0.077), due to studying a relatively similar number of larvae. The number of pupae differed significantly (ANOVA; df=3, F=78.974, P=0.00), also the percentage of pupation (ANOVA; df=3, F=110.128, P=0.00). Significant differences were detected between control group and all the treatment groups (Table 3). This emphasizes the negative impacts of treated water with Altosid on honey bee larvae. The treatment with A3W caused the highest reduction in the pupation percentage followed by A2W, and finally A1W. A3W differed significantly than A1W, while A2W showed insignificant differences than A1W and A3W (Table 3).

Table 3 Numbers and percentages as means± S.E. of honey bee larvae and pupae impacted by Altosid. Water collected after one (A1W), two (A2W), and three weeks (A3W) post-treatment with Altosid. Means marked with the same letter in the same column are not significantly different according to Tukey test.

Treatment	Number of treated larvae	Number of pupae	Percentage of pupation
A1W	63.00±1.73a	18.33±2.96b	29.32±5.43b
A2W	70.00±1.15a	10.67±1.45bc	15.27±2.22bc
A3W	70.67±1.45a	4.00±1.52c	5.66±2.14c
Control	66.67±2.90a	55.67±3.71a	83.35±2.15a

3.2.2 Survival of workers

The cumulative survival of honey bee workers belong to the studied groups over the study period is shown in Fig.6. The survival was high to sugar syrup group (control group) followed by A1W, A2W and A3W, respectively. The estimated survival mean was 9.41±0.23, 9.35±0.25, 8.86±0.28, and 8.43±0.31 days for control group, A1W, A2W, and A3W, respectively. There was no significant difference between control group and A1W group (Mantel-Cox test= 1.68, P=0.194>0.05), but significant differences between control group and A2W (Mantel-Cox test= 4.34, P=0.037<0.05) and A3W (Mantel-Cox test= 16.17, P=0.0001<0.05) were found.

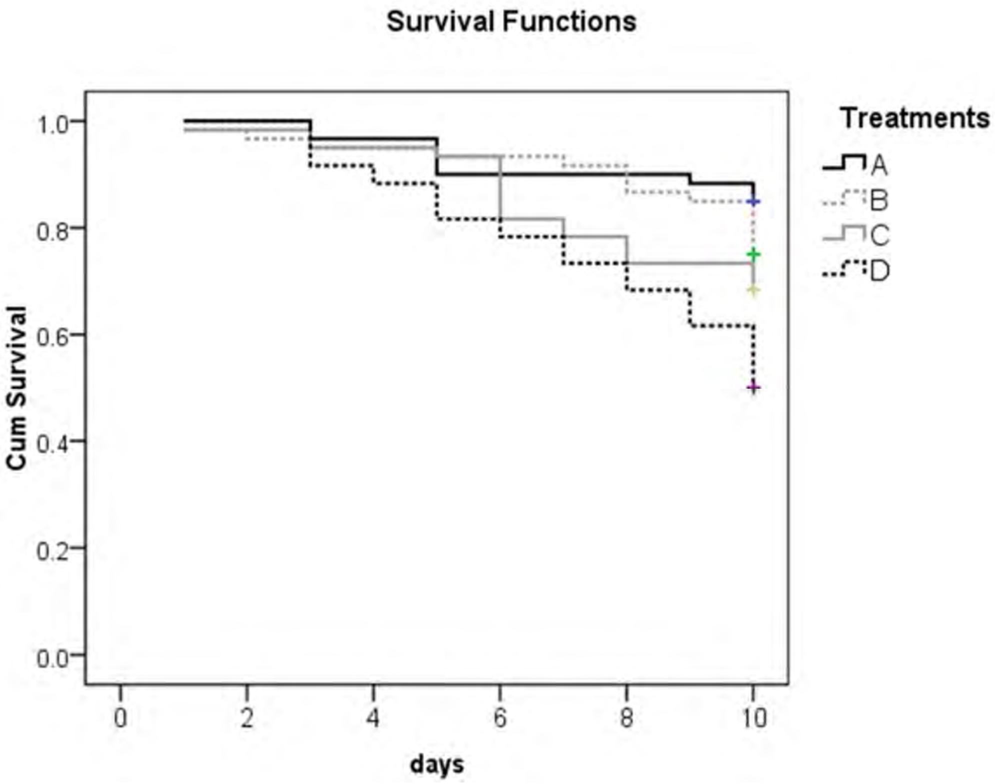

Fig. 6 The cumulative survival of honey bees over 10 days for sugar syrup only (A), sugar syrup prepared using water collected after one (B), two (C), and three weeks (D) post-treatment with Altosid.

4 Discussion

4.1 Efficacy period of Altosid against mosquito

For the both districts, Altosid showed efficacy in preventing adult formation with percentages above 90% for 43 to 45 days (about 6 weeks), and this efficacy decreased over time. Similarly, emergence of *Culex* mosquitoes reduced by 70% due to methoprene (Altosid) after 15 weeks under field conditions and by 99% after 12 weeks under laboratory conditions (Knepper et al., 1992), and reduced by 82% due to Altosid for a period of 15 weeks under field conditions (McCarry, 1996). Also, Altosid effectively controlled mosquitoes for a month under field conditions and substantially longer under laboratory conditions (Butler et al., 2006), and fell below the minimum lethal concentration after one or two weeks after treatment (Des Lauriers et al., 2006). The obtained results from the present study and the previous studies, generally, support the ability of Altosid to impact mosquito development negatively over relatively long period. The variation between the reported periods of efficacy between this study and the previous studies can be explained by the variations in the experimental conditions.

The trend of larval mortality at the end of each test differed between the two districts, and percentages of inhabited adults differed significantly between the two districts during the first 4 tests. These changes may indicate that Altosid efficacy was impacted by water type (i.e. the presence of organic matter or suspended soil particles which result in the removal of the Altosid from the water). But such changes in water type are not high because the highest efficacy period for Altosid in water from the two districts was similar and up to 45 days, and water without treatments from the two districts showed similar adult formation percentages. Over the 8 tests the recorded parameters for larvae reared in water from the two districts showed the absence of any significant differences, supporting the few impacts of water type on Altosid efficacy.

4.2 Effects of Altosid on honey bees

4.2.1 Immature stages

The bees removed some of the treated larvae while those succeeded to pupate were able complete the development successfully. The treated larvae with water collected after one, two, and three weeks post-treatment with Altosid showed significant negative effects on the pupation rate than the control group. This highlights the impacts of the tested IGR on honey bee larvae. The negative impacts on bee larvae could be explained by the role of methoprene to impair the development. A previous study showed that Altosid eliminated brood production (Barker and Waller, 1978), and this is in line with the present study. Other IGRs than Altosid showed reduction in brood production (Amir and Peveling, 2004; Thompson et al., 2005), and the diflubenzuron, an IGR, under laboratory conditions, caused larval mortality from 47.2 to 63.9% of the treated honey bee larvae significantly higher than the control (Dai et al., 2018). These studies together with the present study confirm the harmful effects of IGRs on immature stages of honey bees.

4.2.2 Survival of workers

Feeding bees on syrup prepared from water collected after two or three weeks post-treatment with Altosid significantly impacted the survival of the bees negatively. This reflects the potential negative effects of methoprene on adults of honey bees. For only one week showed no significant impacts than sugar syrup. According to (Barker and Waller, 1978) Altosid did not impact bee mortality, this finding is in line with the results obtained for treatments with syrup prepared from water collected after one week post-treatment with Altosid but not after two or three weeks. However, the possible effects of IRGs on adult bees are supported by previous studies. Previous work using other IGRs than Altosid showed reduction in flight activity of honey bees (Amir and Peveling 2004). Negative impacts of IGRs were recorded on the survival of bee workers and hence on colony size and viability (Thompson et al. 2007), and on queens and absconding trend (Milchreit et al., 2016). Additionally, two IGRs (methoxyfenozide and pyriproxyfen) can cause negative effects on survival of foragers (Fisher et al., 2018). These studies support the negative impacts of IGR on honey bee adults as found in the present study, which may be occur due to some physiological impacts. From experiments on mosquito and honey bees, it is clear that negative effects on these organisms are high during the first 21 days post-treatment of water with Altosid. However, high negative effects were found on mosquito larvae during the first week post-treatment with Altosid unlike honey bees. This happened because mosquito larvae were reared in treated water and were in continues exposure to Altosid while honey bees were just treated with dissolved Altosid in water. Thus, the negative impacts on honey bees increased when Altosid was dissolved in water for three weeks (i.e. high concentration of Altosid in water).

5 Conclusion

It can be concluded that the use of Altosid XR-briquets (methoprene as an active ingredient) as sustained-release formulation may result in more effective control against mosquito larvae over extended periods up to 45 days using a single application, hence reducing labor costs since less frequent application are required. So, such formulations may be particularly useful for use in any location near the household areas where pools of water may remain for long periods. Also, the source of pond water used for rearing larvae showed no impact on the overall period of the highest efficacy of Altosid. Concerning honey bees, the pupation percentages of larvae treated by water collected after one, two and three weeks post-treatment with Altosid declined significantly than the control group. The survival of bee workers impacted negatively than the control group when fed on syrup prepared using water collected after two and three weeks post-treatment with Altosid. This suggests the potential hazard of Altosid on bee colonies. Thus, beekeepers are advised to place their colonies away from treated areas.

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