Toxicological and biochemical studies for chlorpyrifos insecticide on some mosquito larvae and their associated predators

Shaimaa H. Mohammed, Randa I. Eltaly and Hend H. Salem

Zoology Department, Faculty of Science, Al-Azhar University (Girls Branch), Cairo, Egypt

ABSTRACT
Use of chemical insecticide with natural enemies could be more effective in mosquito control strategy. This study examined the toxicity of chlorpyrifos insecticide against three field-collected mosquito species, *Culex pipiens*, *Anopheles pharoensis* and *Ochlerotatus caspius* and their associated predators dragonfly (*Pantala flavescens*) and mayfly (*Caenis stephens*) naiads as non-target insect for chlorpyrifos. The predation potential of *Pa. flavescens* and *Ca. stephens* against tested mosquito larvae was also investigated. Additional biochemical assays were carried out to detect the effect of chlorpyrifos on some detoxifying enzymes of tested mosquito larvae and their associated predators. The results showed that (*Pa. flavescens*) have higher predation potential than (*Ca. stephens*). The toxicological results recorded high toxic effect of chlorpyrifos on *Ca. stephens* followed by *An. pharoensis* and *Oc. caspius* with percent mortality 100, 90 and 85% respectively, while *Pa. flavescens* exhibited high resistance followed by *Cx. pipiens* with percent mortality 20 and 40% respectively. Moreover, Acetylcholinesterase and glutathione S-transferase were significantly increased only in *Cx. pipiens* and *Pa. flavescens*. It could be concluded that, chlorpyrifos have different toxicological effect on the tested mosquito larvae and associated predators. So, the side effect of chlorpyrifos must be taken in consideration before using it in control programs.

Introduction
Mosquitoes was deemed to be one of the most medically and economically important insects. They are predominant, and able to transmit a wide variety of human and animal diseases [1,2]. These diseases cause for millions of deaths yearly [1,3].

Mosquitoes have many natural enemies such as invertebrate predators which play important roles in reducing the mosquito larval populations. Natural predators distinguish by restricted breeding season and prolonged generation period than that of mosquito larvae, so they have a necessary role in mosquito larva control. Odonata (dragonfly) is one of the common predators for mosquito controls. Dragonflies plays important role in mosquito control for many years ago, also considered as one of the most arthropods for to mosquito control [4].

Some species of mayfly naiads (Ephemeroptera) are carnivorous, but most of the naiads feed on algae, higher plants, and organic material [5]. However four North American mayfly naiads appear to be carnivorous [6]. The carnivorous habit and

CONTACT Shaimaa H. Mohammed shimaahamed428@gmail.com Zoology Department, Faculty of Science, Al-Azhar University (Girls Branch), Cairo, Egypt

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its adaptive changes appear to have had at least three species among the four genera of North American mayflies [7]. The predation efficiency of mayfly naiads in Egypt weren’t examined on mosquitoes.

Chemical insecticides still considered the most important methods for mosquito population’s depression [8]. Insecticides are threatening the environment; the effect of insecticides on aquatic systems may be more common and more important than direct effects on pest [9]. There are less predictable effects of insecticides in non-targets populations in the same mosquito breeding habitat. The potential effects of insecticides on non-target organisms can vary widely depending on their mode of action and insect species [10].

This study investigated the predation efficacy of dragonfly and mayfly on mosquito larvae and the effect of chlorpyrifos insecticide on target mosquitoes and their non-target predators, as well as the activity of the most important detoxifying enzymes acetylcholinesterase and glutathione-S-transferase of both mosquito larvae and their predators were examined.

**Materials and methods**

**Study area**

Wild populations of mosquitoes were collected from different mosquito breeding sites; brackish water, water pool, rice field and drainage water during the study from El Fayoum Governorate (a large oasis in Western Desert); El-Galaa, (29°21′22.6″N 30°40′50.7″E), Tameyah city, Tamiyyah district (29°28′19.7″N 30°57′04.1″E) and El Nazlah (29°18′54.6″N 30°38′33.6″E) Ibsheway district.

**Larvae collection and identification**

To promote mosquito larvae collection, the breeding site was examined for the presence of larvae by dipping using a 250 ml standard mosquito larvae dipper according to WHO, [11] method. The physicochemical parameters of breeding sites were measured such as; salinity, total dissolved solid, pH, conductivity and temperature. The collected larvae were transferred into labeled plugged plastic jars, leaving 3 cm space for larvae breathing and every two hours plug was removed to supply the specimens with fresh air until reaching the lab [11].

Larvae were inspected and identified according to [12,13]. The most abundant *Culex pipiens*, *Anopheles pharoensis* and *Ochlerotatus caspius*. Sufficient numbers of living larvae were used for predation experiment and larval bioassay. On the other side, treated larvae with chlorpyrifos were stored frozen till biochemical assays.

**Collection of mosquito predators**

Mosquito predators were collected from the same mosquito larval breeding habitats. The collected insects were listed and transferred to the lab in sampling jars provided with water from the same collected sites. Associated insects to mosquito larvae were identified to family and species according to Bouchard, [14–16]. The two most abundant predator species were then used for the predation experiment and also bioassays and biochemical assays.

**Predation potential trial**

Predation efficiency of the dragonfly and mayfly naiads was investigated according to [17]. Predators were provided with tested mosquito larvae and pupae as supplementary food. Four replicates of 100 larvae of each instars as well as pupae separately were provided to one dragonfly or mayfly naiads within the beakers of 500 ml capacity with only 300 ml H2O. Monitoring prey mortality was investigated by calculating and recording of live mosquito larvae and pupae at an interval of 6 and 24 h.
Toxicological studies

Bioassay for target mosquitoes and non-target predators

Chlorpyrifos, which recommended for mosquito larval control [18] was used in the present investigation. Chlorpyrifos was bought from the Medical Entomology Institute, Doki; Egypt. Single-concentration (diagnostic dose 0.01 ppm) [19], as conducted against the three mosquito species, as well as their predators (dragonfly and mayfly naiads). The bioassay method was according to WHO procedure [20]. Four replicates with 25 from 3rd instar of each tested mosquito larvae, as well as predator naiads per replicates were put in glass beaker contained 249 ml tap water and 1 ml of the chlorpyrifos diagnostic dose. The mortality was recorded after 24 hours post treatment. Other four replicates were treated with only 1 ml ethanol as control experiment and mortality never exceeded 4%.

Biochemical experiments

Specimens preparation

Biochemical assays were conducted after 24 h after chlorpyrifos exposure to determine the activity of some detoxifying enzymes (acetylcholinesterase and glutathione S-transferase) in the tested species. Specimens were prepared by homogenization of 10 individuals in an ultrasonic homogenizer, and then the homogenates were centrifuged for 15 min in a cooling centrifuge [5°C, 8000 r.p.m]. The final supernatants were kept frozen till used [21].

Enzymes assay

Total proteins activity was estimated as stated by [22], by using Bio-Rad protein assay, while Glutathione S-transferase [GST] activity was determined as stated by [23], by using (1-chloro-2, 4dinitrobenzene) as substrate. Finally acetylcholinesterase [AChE] activity was calculated as stated by [24], using acetylcholine bromide [AChBr] as substrate.

Statistical analysis

Mortality data were pooled from four replicates and figured by using Microsoft Excel (Microsoft office, 365). Predation and biochemical assay were gathered from three replicates and determined as (mean± SD & mean ± SE, respectively). The data were statistically analyzed by ANOVA using SPSS V. 20 [25].

Results

Survey of mosquito species

Mosquito larvae were found in different breeding habitats; drainage water, brackish water, rice field and water pool. The results showed that the occurrence of four mosquito species in the study area: two Culicines, Culex pipiens (Linnaeus), Cx. antennatus (Becker), while Culex pipiens was the most abundant one. As well as there was one Anopheline, An. pharoensis and one Aedine, Oc. Caspius (Table 1).

Survey of associated mosquito predators

The two collected naiads; dragonfly: Pantala flavescens (Odonata: Libellulidae) and mayfly: Caenis stephens (Ephemeroptera: Caenidae), were inhibited the shallow, brackish and highly polluted water which contain high dissolved oxygen, alkaline pH, high organic materials, high dissolved solid and high conductivity. They were also associated with mosquito larvae in breeding places studied. Moreover, mosquito larval predators prefer polluted water than fresh water. In general dragonfly naiads were collected in large number than mayfly naiads (Table 1).
Table 1. Predominance of mosquito larvae from different breeding sites in El-fayoum.

| species | Culex Pipiens | Culex antennatus | Anopheles pharoensis | Ochlerotatus caspius | Caenis spp | Pantala flavescens | T (°C) | pH | DO (mg/L) | TDS (mg/L) | EC (µS/cm) |
|---------|---------------|------------------|----------------------|---------------------|------------|-------------------|--------|----|----------|-----------|-----------|
| 1       | 1304          | 59               | 2139                 | 1110                | 50         | 67                | 19.9   | 8.1| 14.1     | 10.9      | 1.81      |
| 2       | 1150          | -                | 15                   | 1220                | 40         | 12                | 18     | 8.3| 26.5     | 1.02      | 1.82      |
| 3       | 892           | 15               | 341                  | 890                 | -          | 25                | 17.5   | 7.8| 25.1     | 1.01      | 2.11      |
| 4       | 225           | 11               | 111                  | 220                 | 13         | 34                | 15.8   | 8.5| 14.4     | 1.24      | 2.48      |
| Total No. | 3571        | 85               | 2958                 | 3440                | 103        | 138               | -      |    | -        | -         | -         |

T. Temperature, pH. Potential hydrogen, Do. Dissolved oxygen, TDS. Total dissolved solid, EC. Electrical conductivity.

Predation potential

Measurement of predation potency of dragonfly and mayfly naiads on mosquito immature stages of three tested species (Cx. pipiens, An. pharoensis and Oc. caspius) indicates that, dragonfly naiads have greater predation efficiency than mayfly naiads. The result showed also that tested predators appeared to like better the youngest larval instars of mosquito (Table 2, 3 and 4).

The results indicate that, mayfly naiads prefer mainly An. pharoensis larvae followed by Oc. caspius, while Cx. pipiens was showed the least predation rate. There wasn’t any predation of mayfly naiads at the first 6 hours on all instars of tested mosquitoes predator potency equal zero (didn’t be represented in table), while at 24 hours the maximum predation of mayfly naiads was on the 1st instar larvae only (18, 15.3 & 13.5 for An. pharoensis, Oc. caspius Cx. pipiens, respectively) followed by the 2nd and 3rd larval instar (11.8 & 8.5) and (12 & 9.3), (11.5 & 7.8), for An. pharoensis, Oc. Caspius and Cx. pipiens, respectively. The minimum predation was on the 4th larval instar (1, 2 & 0, for An. pharoensis and Oc. Caspius and Cx. pipiens, respectively, while there wasn’t any predation in pupal stage of the three tested mosquito species preys (Table 2).

The predation rate of dragonfly naiads showed that naiads prefer mainly Cx. pipiens followed by An. pharoensis then Oc. Caspius at 6 h, while at 24 h An. pharoensis recorded the higher predation rate. The feeding data of dragonfly after 6 h (Table 3), showed that, the 3rd, 2nd and 1st recorded the highest predation ratio (7.8, 9.5 & 6), (8, 7 & 8) and (6.8, 3.5 & 4) for Cx. pipiens, An. pharoensis and Oc. caspius, respectively. On the other hand, the 4th larval instar was the lowest favorable prey recorded (3.5, 3.5 and 5.8,) for Cx. pipiens, An. pharoensis and Oc. caspius, respectively. Again there wasn’t any

Table 2. Predatory potency of caenis stephens (mayfly) naiads over the mosquito larvae and pupae of three mosquito species after 24 h.

| Mosquito species prey | 1st larval instar | 2nd larval instar | 3rd larval instar | 4th larval instar | Pupal |
|-----------------------|-------------------|-------------------|-------------------|-------------------|-------|
| Anopheles pharoensis  | 18 ± 0.82         | 11.8 ± 1.3        | 8.5 ± 1.3         | 2 ± 0.82          | 0 ± 0 |
| Ochlerotatus caspius  | 15.3 ± 1          | 12 ± 0.82         | 9.3 ± 1           | 1 ± 0.82          | 0 ± 0 |
| Culex pipiens         | 13.5 ± 1.3        | 11.5 ± 1.3        | 7.8 ± 1.7         | 0 ± 0             | 0 ± 0 |

Table 3. Predatory potency of pantala flavescens (dragonfly) naiads over the mosquito larvae and pupae of three mosquito species after 6 hrs.

| Mosquito species | 1st larval instar | 2nd larval instar | 3rd larval instar | 4th larval instar | Pupal |
|-----------------|-------------------|-------------------|-------------------|-------------------|-------|
| Culex pipiens   | 6 ± 0.82          | 9.5 ± 0.58        | 7.8 ± 0.96        | 3.5 ± 0.58        | 0 ± 0 |
| Anopheles pharoensis | 8 ± 0         | 7 ± 0.82          | 8 ± 0.82          | 3.5 ± 0.58        | 0 ± 0 |
| Ochlerotatus caspius   | 4 ± 0.82         | 3.5 ± 0.58        | 6.8 ± 0.96        | 5.8 ± 0.96        | 0 ± 0 |
predation in pupal stage of three mosquito species. The results also indicate that, at 24 hours the maximum predation of dragonfly naiads was on the 3rd followed by 2nd then 1st larval instar (50, 37.8 & 32.5), (46.8, 42 & 40.3) and (39.5, 30.3 & 33.3) for Cx. pipiens, An. pharoensis and Oc. caspius, respectively. Again the 4th recorded the lowest predation ratio followed by pupal stage (17.5 & 9.8), (21 & 6.5) and (14.5 & 8.3) for Cx. pipiens. An. pharoensis and Oc. caspius, respectively (Table 4).

Susceptibility of mosquito larvae and associated aquatic predators to chlorpyrifos insecticides

Data of tested of mosquito larval species; Cx. pipiens. An. pharoensis and Oc. caspius as target for chlorpyrifos in Figure 1 showed that An. pharoensis larvae were more susceptible to chlorpyrifos diagnostic dose with percent mortality + SE (90%+ 2), followed by Oc. caspius with percent mortality + SE (85% + 2). While Cx. pipiens larvae have high resistance to chlorpyrifos with percent mortality (40% + 1.8).

Whereas susceptibility to chlorpyrifos in its non-target predators varied depending on the species (Figure 1). Caenis stephens (mayfly naiads) showed high susceptibility to chlorpyrifos with percent mortality 100%, while Pa. flavescens (dragonfly naiads) recorded high resistance to chlorpyrifos with percent mortality + SE (20% +1).

Table 4. Predatory potency of pantala flavescens (dragonfly) naiads over the mosquito larvae and pupae of three mosquito species after 24 h.

| Mosquito species       | 1st larval instar | 2nd larval instar | 3rd larval instar | 4th larval instar | Pupal |
|------------------------|-------------------|-------------------|-------------------|-------------------|-------|
| Culex pipiens          | 32.5 ± 2.1        | 37.8 ± 2.23       | 50 ± 1.83         | 17.5 ± 1.73       | 9.8 ± 1.71 |
| Anopheles pharoensis   | 40.3 ± 1.3        | 42.8 ± 0.82       | 46.8 ± 1.5        | 21 ± 1.8          | 6.5 ± 1.3 |
| Ochlerotatus caspius   | 33.3 ± 3.9        | 30.3 ± 7.4        | 39.5 ± 1.3        | 14.5 ± 1.3        | 8.3 ± 0.95 |

Biochemical assay

In order to realize the base on which resistance of the mosquito larvae and their tested natural enemies to chlorpyrifos and activities of two detoxified enzymes involved in resistance mechanism; acetylcholinesterase (main target sit of

Figure 1. Toxicity of the chlorpyrifos insecticide against mosquito larvae and associated aquatic predators.
chlorpyrifos) and glutathione S-transferase were evaluated. The comparison of the two enzymes levels between treated and untreated (control) populations were investigated. The total protein was measured in all tested species; the values were used to standardize the activity of AChE and GST enzymes. Caenis stephens showed mortality 100%, so the biochemical assay was carried only for the other tested species (Cx. pipiens, Oc. caspius, An. pharoensis and Pa. flavescens). The results of AChE and GST were represented in Table 5 & 6. In general, there is great elevation in the levels of AChE than those of GST after treatment of chlorpyrifos as compare with control ones. Represented data showed that, there is significant increase in the activity of AChE in Cx. pipiens larvae and Pa. flavescens naiads after treatment with chlorpyrifos (6.1 ± 0.15 & 5.4 ± 0. 3 μg AChBr/min/mg protein, respectively), as compared to control which recorded (2.94 ± 0.11 & 3.6 ± 0.1 μg AChBr/min/mg protein, respectively). While there is no significant increase in the levels of AChE in Oc. caspius, An. pharoensis after treatment with chlorpyrifos (2.2 ± 0.2 & 1.3 ± 0.15 μg AChBr/min/mg protein, respectively) in compared with control which recorded (1.38 ± 0.1 & 0.8 ± 0.1 μg AChBr/min/mg protein, respectively) (Table 5).

There is a significant increase in the levels of GST (Table 6), in Cx. pipiens larvae and Pa. flavescens naiads after treatment with chlorpyrifos (3 ± 0.11 & 3.62 ± 0.1 mmol sub. conjugated/min/mg protein, respectively), as compared to control which recorded (2 ± 0.12 & 2.7 ± 0.13 mmol sub. conjugated/min/mg protein, respectively), while there is no significant increase in the level of GST and in Oc. caspius, An. pharoensis after treatment with chlorpyrifos (2 ± 0.1 & 1.78 ± 0.1 mmol sub. conjugated/min/mg protein, respectively).

**Discussion**

The chemical insecticides considered an effective master plan in preventing the mosquito-borne diseases. There are very little insecticides that recommended for mosquito control from
WHO. Unfortunately, mosquito species have advanced resistance mechanisms to most insecticide groups [18,26].

The mosquito control strategy required alternative methods that are ecofriendly [27]. Biological control strategies considered one of the most ecofriendly, prospective and targeting mosquito species [28].

The present study detects gluttonous and intensive predation of dragonfly naiads (Pantala flavescens) on all tested mosquito species, while mayfly (Caenis stephens) recorded lower predation efficiency than dragonfly naiads, also the two tested predators preferred the youngest stages. The present results agree with [29] who reported that the predation potency of dragonfly naiads caused 50 reductions in Culiseta longiareolata breading site. Odonata naiads are large that requires large space and might polyphagous and might be in efficient for mosquito larvae. The dragonfly naiads were active and strong predators for mosquito larvae, especially An. pharoensis [30]. As well as mayfly naiads consider herbivores, which feeds on algae, or detritivores – robbing waste of flooded leaves and stones. A few species can prey on minute animals [31]. Also [32], estimated that the dragonfly naiads (Labellula spp.) prefer to consume all stages of Aedes aegypti larvae and pupae but prefer the smaller one [33], reported that Pa. flavescens naiads are excellent predator and able to consume Ae. aegypti larvae in large quantities in lab conditions.

In the current study, the toxicity of chlorpyrifos insecticide was tested against three mosquitoes (Culex pipiens. Anopheles pharoensis and Ochlerotatus caspius) species as well as their predators (Caenis stephens and Pantala flavescens), the result showed that, An. pharoensis larvae were more susceptible to chlorpyrifos followed by Oc. caspius with percent mortality 90%, and 85%, respectively. On the other hand, Cx. pipiens larvae have high resistance to chlorpyrifos with percent mortality 40%. Effects of chlorpyrifos on mosquito associated predators also have been tested. The result evaluated that, chlorpyrifos is a slightly toxic to dragonfly naiads Pa. flavescens. In contrast, chlorpyrifos is a highly toxic to mayfly naiads Ca. stephens with percent mortality 100%.

The effect of chlorpyrifos in target mosquitoes agrees with [34], who examined the susceptibility of Cx. quinquefasciatus larvae to chlorpyrifos, he found that Cx. quinquefasciatus larvae are more resistant to chlorpyrifos. Also, [35], observed that An. gambiae s.l was fully susceptible to chlorpyrifos and fenitrothion but resistant to DDT and pyrethroids. As well as [36], who evaluated the effect of chlorpyrifos on six strains of Aedes aegypti. Relative to LC50 and LC90, three strains were highly resistant to chlorpyrifos, one strain was sparingly resistant, and two strains had high susceptibility.

Effect of chlorpyrifos on mosquito predators agree with [37], who observed that susceptibility to spinosad differed between predator insects; Caenis spp. was the most susceptible, while Ischnura sp. and Pa. longipennis showed low susceptibility. [38], found that toxicity of most insecticides to aquatic organisms is due to share the same mode of action as insects, and also to lack proper detoxification systems. [39], expected that pyrethroids high toxicity to mayfly, dragonfly naiads, followed by neonicotinoids, as well as aquatic insects are more sensitive to organophosphorus than carbam-ates.

To gain satisfactory explanation for effect of chlorpyrifos on mosquito larvae and its predators, a biochemical assay was investigated for two enzymes which are known to be involved in insect resistance. The enzymes are acetylcholine esterase AChE and glutathione S-transferase GST.

Chlorpyrifos is an organophosphorus insecticide, which inhibiting the breakdown of acetylcholine. When insects are exposed, chlorpyrifos interacts to the active site of the AChE, which inhibiting breakdown of
acetylcholine in the synapse [40]. Elevation of AChE in resistant insects results in a decreased sensitivity [41]. Glutathione S-transferase (GST) which is dimeric multifunctional enzymes involved in detoxification of a large range of xenobiotics. Elevated GST activity leads to increase resistance of insect to different classes of insecticides [42].

The results of biochemical assays detected high elevation in the activity of AChE and GST especially in Cx. pipiens and Pa. flavescens is supported the increase chlordane resistance in tested population.

The results agree with [43], who suggested that occurrence of OP have great risk to all aquatic environments. Inhibition (AChE) is a specific target site to both OP and carbamate insecticides, like OP pesticide chlordane, aldicarb also affects acetylcholinesterase activity [44]. As well as, GST are most important detoxifying enzymes [45], so the mode of action of insecticides is responsible the toxicity degree to all organisms. Aquatic insects usually have high susceptibility to most types of insecticides because they have many physiological features share with the target insects [39].

Conclusions

t could be concluded that, dragonfly naiads have high predation efficiency against all tested mosquito species, and also mayfly naiads can considered as predators for mosquito larvae. Chlordane insecticide has different degrees of toxicity on non-target mosquito natural enemies. So, we must be taken in consideration the side effect of insecticides before used it in control programs.

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ORCID

Shaimaa H. Mohammed http://orcid.org/0000-0003-3436-6812

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