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Activity of spiromesifen on growth and development of *Culex pipiens* (Diptera: Culicidae): Toxicological, biometrical and biochemical aspects

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Abstract
The present study aimed to evaluate the activity of spiromesifen, an insecticide belonging to the chemical group of spirocyclic tetronic/tetramic acid derivatives, against the most abundant and investigated mosquito species, *Culex pipiens* Linné, 1758 (Diptera, Culicidae). A commercial formulation of spiromesifen (Oberon^{R} 240 SC) was tested at different concentrations ranging between 238 and 1428 µg/L on newly molted fourth-instar larvae under standard laboratory conditions according to WHO recommendations. The effects were examined on the mortality, the morphometric measurements and the biochemical composition of body of larvae, respectively. The compound exhibited insecticidal activity and mortality occurred after earlier inhibition of their development or by their inability to complete their ecdysis. Moreover, it disturbed growth and several morphological aberrations were observed. It was also found to affect body volume, contents of carbohydrates, lipids and proteins, and their caloric indexes. Spiromesifen exhibited insecticidal activity against *C. pipiens* and affected the main biochemical components of body with a marked effect on lipids and malondialdehyde confirming its primary mode of action on lipid synthesis. However, it appears less potent than other insecticides tested such as the insect growth disruptors.

Keywords: Mosquitoes, *Culex pipiens*, spiromesifen, toxicity, morphometry, biochemistry

Introduction
Vector control is an essential requirement in control of epidemic diseases that are transmitted by mosquitoes [1]. These diseases that cause morbidity, mortality, economic loss, and social disruption is well-documented [2, 3]. The house mosquito *C. pipiens* Linné 1758 (Diptera: Culicidae) is the most widely distributed mosquito species [4] and plays an important role in diseases transmission [1]. This species is known to carry arboviruses (arthropod-borne viruses) and is recognized as the primary vector of St. Louis encephalitis and West Nile Virus [5]. *C. pipiens* is the most abundant mosquito species in Algeria [6, 7, 8]. However, these conventional neurotoxins possess strong secondary effects on the environment [9, 10]. Spiromesifen is a systemic insecticide/ acaricide belonging to the class of spirocyclic tetronic/tetramic acid derivatives. It acts on lipid synthesis by inhibiting acetyl CoA carboxylase [11] and causes a significant decrease in total lipids [12, 13]. This compound has been introduced in several countries over the last few years and is becoming an important compound for controlling whiteflies and mites in resistance management programmes, along with other effective insecticides such as neonicotinoids and diafenthiuron. Several recent studies have shown the effectiveness of spiromesifen against a variety of insect pests [11, 14]. The carbohydrates play a crucial role in the physiology of insects and the rates of glycogen in tissues are closely related to the physiological events such as the flight, the moult and the reproduction [15]. In addition, lipids play an important role in general metabolism and reproduction [16]. Moreover, the fatty acids constitute precursors of cuticular hydrocarbons and pheromones [17]. Malondialdehyde (MDA) a product of lipid peroxidation has been widely used as a marker of free radical damage to lipid molecules [18].
Previously, we reported that spiromesifen was found to reduce the amounts of body lipids and to enhance the rate of MDA in *Drosophila melanogaster* pupae [13]. In the present study conducted under laboratory conditions on *C. pipiens*, a medically important mosquito species, we assessed the potency of spiromesifen against fourth-instar larvae by determining the lethality parameters.

In a second series of experiments we investigated the metabolic responses following spiromesifen exposure by measuring catalase (CAT) activity and MDA rate, biomarkers of oxidative stress and lipid peroxidation, respectively. In addition, its effects on morphometric measurements and on main biochemical components (carbohydrates, proteins and lipids) and caloric indexes in whole body were investigated. The data obtained provide better insights on its mode of action and give information on its potential for use as a mosquito control agent.

Materials and Methods

Mosquito rearing

Culex pipiens eggs and larvae were collected in 2013, from untreated areas located at Tébessa (Northeast Algeria). Larvae specimens were morphologically identified according to Brunhes *et al.* (1999) [19] and kept as previously described [20]. Pyrex storage jars (80 by 100 mm) containing 150 ml of tap water were maintained at temperature 25°C and a photoperiod of 14:10 (L: D). Larvae were daily fed with fresh food consisting of a mixture of Biscuit Petit Regal-dried yeast (75:25 by weight), and water was replaced every four days.

Toxicity bioassays

The insecticidal assay was conducted as previously described [9]. A trade formulation of spiromesifen (Oberon^{R} 240 SC, Bayer Crop Science) courtesy of Pr. G. Smagghe (Ghent University, Belgium) was added to treatment beakers at different final concentrations (238, 476, 714, 952 and 1428 µg active ingredient per liter). Newly molted fourth-instar larvae of *C. pipiens* (< 8 h) were exposed to the different concentrations for 24h in accord with World Health Organization (WHO) criteria [21]. Controls were exposed to water only. After the exposure time of 24 h, larvae were removed, washed with untreated water and placed in clean water. The test was carried out with 4 replicates containing each 25 larvae per concentration. Growth was examined and mortality was registered daily until adult emergence. The mortality percentage obtained was corrected [22] and toxicity data were studied by probit analysis [23]. Lethal concentrations (LC_{50} and LC_{90}) and 95% confidence limits (95% CL) were estimated, and slope of the concentration-mortality lines were calculated [24].

Determination of catalase activity and malondialdehyde rate

Catalase (CAT) activity was measured by determining the decomposition of its substrate H_2O_2 as described by Claiborne (1985) [25]. Each sample (3 pools each containing 10 individuals) was conserved in buffer phosphate (100 mM; pH 7.4). After sonication and centrifugation (15 000 rpm for 10 min), the supernatant was collected and used for the determination of the CAT activity. The protein amount in the total homogenate was quantified according to Bradford (1976) [26]. The absorbance was red at 240 nm. The assay was conducted with 6–8 repeats and data expressed as µmol/min/mg protein.

The concentration of malondialdehyde (MDA) was determined as lipid peroxidation index according to Draper & Hadley (1990) [27] as previously described [13]. This method was based on a spectrophotometric measurement of the reaction of thiobarbituric acid (TBA) with MDA at 532 nm. The protein content was evaluated according to Bradford (1976) [26] using bovine serum albumin as standard (BSA, Sigma). The rate was expressed as µmol/mg protein.

Morphometric measurements

As above, newly moulted fourth instar larvae were treated with spiromesifen at its LC_{50} and LC_{90} as determined before. The morphometric measurements were performed following the procedure of Timmermann & Briegel (1998) [28]. The body volume corresponds to cubic value of width.

Biochemical composition of body

Protein, carbohydrate and lipid were extracted following the procedure of Shibko *et al.* (1966) [29] and quantified as previously described [30]. Newly molted larvae were collected. Pooled samples (10 individuals per pool) were weighed and extracted in 1 ml of trichloracetic acid (20%). In brief, quantification of proteins was carried following the Coomassie Brilliant Blue G-250 dye-binding method [26] with bovine serum albumin as a standard. The absorbance was measured at 595 nm. Carbohydrates were determined according to Duchateau & Florkin (1959) [31] using anthrone as reagent and glucose as standard. Lipids were measured by the vanillin method [32] and the table oil (99% triglycerides) used as a standard. Data were expressed in µg per individual and assays conducted with 3 replicates per treatment.

Determination of caloric indexes

The caloric index (CC) per individual was performed as follow. The different contents for total proteins, lipids, or carbohydrates were converted into calories: 1 calorie corresponds to 0.004 µg of carbohydrates and proteins, whereas for lipids 1 calorie corresponds to 0.009 µg [33] as previously described [34]. Caloric values of proteins, lipids, or carbohydrates were related to body size. The size specific caloric content (SSCC was claculated according to Briegel & Timmermann (2001) [16]. All parameters were calculated from 3 pools each containing 10 individuals for each stage.

Statistics

The number of individuals tested in each series is given with the results. Data are presented as the mean ± standard deviation (SD).

The significance between different series was tested using Student's t test. All statistical analyses were performed using MINITAB Software (Version 16, PA State College, USA) and $p \leq 0.05$ was considered to be a statistically significant difference.

Results

Insecticidal activity

Dose-response relationship was determined for spiromesifen applied for 24h to newly molted fourth instar mosquito larvae. The mortality was scored up to adult emergence. The highest concentration tested (1428 µg/L) caused 96.14 ± 2.57% mortality (Table 1). With probit, LC_{50} was calculated as 542.21 µg/L (FL 95% = 511.72-574.49 µg/L), slope= 4.10 and LC_{90} was 1148.65 µg/L (FL 95%= 998.43-1321.47). Spiromesifen treatment interferes with the pupal-adult development. Examinations of insects after treatment, revealed varying degrees of morphological aberrations (Figs 1-2).

Table 1. Efficacy of spiromesifen on fourth instar larvae of *Culex pipiens*: corrected mortality (mean ± SD, n = 4 repeats each containing 25 individuals).

Doses (µg/L)	238	476	714	952	1428
Mortality (%)	16.06 ± 2.22 a	31.47 ± 1.54 b	52.10 ± 2.09 c	72.75 ± 2.67 d	96.14 ± 2.57 e

Fig 1: Incomplete metamorphosis (larva - pupa) in treated *C. pipiens*

Fig 2: Failed to molt (pupa - adult) in treated *C. pipiens*

Effects on malondialdehyde and catalase

The results summarized in tables 2 and 3 show that the rate of the malondialdehyde (MDA) and catalase (CAT) in control groups increased during the fourth-instar larval stage but in a non-significant manner (p>0.05). In treated series, this increase is significant for the two concentrations tested (LC_{50} and LC_{90}). There was a significant difference between control and treated series at all ages. Indeed, the comparison between control and treated series revealed a significant increase in the rate of MDA at 24 h (control vs LC_{50} p= 0.01 and control vs LC_{90} p= 0.000), 48 h (control vs LC_{50} p= 0.005 and control vs LC_{90} p<0.001) and 72 h (control vs LC_{50} p = 0.001 and control vs LC_{90} p<0.000) (Table 2). Similarly, the increase in the activity of CAT was also significant at 24 h (control vs LC_{50} p= 0.01 and control vs LC_{90} p= 0.000), 48 h (control vs LC_{50} p= 0.005 and control vs LC_{90} p<0.001) and 72 h (control vs LC_{50} p = 0.001 and control vs LC_{90} p<0.000), respectively (Table 3).

Table 2: Effect of spiromesifen (LC_{50} and LC_{90}) on the malondialdehyde rate (µM/mg of proteins) in the fourth instar larvae of *C. pipiens* (mean ± SD, n = 3 pools each containing 20 individuals). Comparison of mean values at different times for a same series (capital letters) and for a same time between different series (lowercase letters).

Time (hours)	Control	LC_{50}	LC_{90}
24	0.09 ± 0.00 a A	0.13 ± 0.00 b A	0.20 ± 0.00 c A
48	0.09 ± 0.01 a A	0.15 ± 0.00 b B	0.23 ± 0.00 c B
72	0.09 ± 0.00 a A	0.17 ± 0.01b C	0.24 ± 0.00 c C

Table 3: Effect of spiromesifen (LC_{50} and LC_{90}) on the catalase activity (µM/mg of protein) in the fourth instar larvae of *C. pipiens* (mean ± SD, n= 3 pools each containing 20 individuals). Comparison of mean values at different time for a same series (capital letters) and for the same time between different series (lowercase letters).

Time (hours)	Control	LC_{50}	LC_{90}
24	3.98 ± 0.14 a A	4.42 ± 0.11 b A	4.76 ± 0.12 c A
48	4.16 ± 0.05 a A	5.08 ± 0.13 b B	6.44 ± 0.12 c B
72	4.19 ± 0.07 a A	5.67 ± 0.08 b C	7.61 ± 0.30 c C

Effects on weight and volume of body

Changes in whole body weight showed a significant reduction in weight of fourth instar larvae (control vs LC_{50} p= 0.038 and control vs LC_{90} p= 0.012). Also, spiromesifen significantly (p= 0.012) reduced the body volume of fourth instar larvae only with the highest concentration (LC_{90}) compared to controls (Table 4).

Table 4: Effect of spiromesifen (LC_{50} and LC_{90}) on the body weight (mg) and the body volume (mm^3) in 4th instar larvae of *C. pipiens* (mean ± SD, n= 3 pools each containing 10 individuals). Mean values followed by the same letter are not significantly different (p>0.05)

Treatment	Control	LC_{50}	LC_{90}
Body weight (mg)	2.70 ± 0.08 a	2.37 ± 0.05 b	2.23 ± 0.05 c
Body volume (mm^3)	3.08 ± 0.05 a	2.36 ± 0.01 a	2.08 ± 0.06 b

Effect of spiromesifen on biochemical composition of bodies

The levels of carbohydrates, lipids and proteins have been estimated in the whole body extracts from fourth larval stage using two lethal concentrations (LC_{50} and LC_{90}). The comparison of mean values shows that the protein content decreased significantly only with the highest concentration (CL_{90}) (control vs LC_{90} p= 0.013). The same effect was observed for the carbohydrate content (control vs LC_{50} p>0.05, control vs LC_{90} p= 0.047). Lastly, the lipid content was reduced significantly with the two tested concentrations (control vs LC_{50} p= 0.027 and control vs LC_{90} p= 0.009).

Table 5: Effect of spiromesifen (LC_{50} and LC_{90}) on contents of proteins, carbohydrates and lipids (µg/individual) in fourth instar larvae of *C. pipiens* (mean ± SD, n= 3 pools each containing 10 individuals). Values followed by the same letter are not significantly different at $p>0.05$.

Treatment	Control	LC_{50}	LC_{90}
Protein content (µg/individual)	50.70 ± 9.30 a	42.90 ± 1.90 a	27.90 ± 2.10 b
Carbohydrate content (µg/individual)	68.44 ± 2.30 a	52.20 ± 2.20 a	48.50 ± 0.90 b
Lipid content (µg/individual)	50.00 ± 2.80 a	39.70 ±2.00 b	30.00 ± 3.70 c

Effect of spiromesifen on caloric indexes

The results mentioned in the table 6 revealed that spiromesifen decreased significantly the caloric contents of both proteins (control vs LC_{50} p= 0.036, control vs LC_{90} p= 0.019) and carbohydrates (control vs LC_{50} p= 0.025, control vs LC_{90} p= 0.007). However, the caloric contents of lipids were affected only with the highest concentration (control vs LC_{90} p= 0.031).

Table 6: Effect of spiromesifen (LC_{50} and LC_{90}) on caloric indexes (Cal) in fourth instar larvae of *C. pipiens* (mean ± SD, n=3 pools each containing 10 individuals). In each line, values followed by the same letter are not significantly different at $P>0.05$.

Treatment	Control	LC_{50}	LC_{90}
Protein caloric index	0.19 ± 0.01 a	0.17 ± 0.01 b	0.11 ± 0.01 c
Carbohydrate caloric index	0.27 ± 0.01 a	0.21 ± 0.01 b	0.19 ± 0.01 c
Lipid caloric index	0.45 ± 0.02 a	0.36 ± 0.02 a	0.27 ± 0.03 b

The specific size caloric indexes (SSCC) of main components (carbohydrates, lipids and proteins) in fourth instar larvae of *C. pipiens* from control and treated series are presented in table 7. Results indicated a significant increase in the SSCC of proteins (CL_{50}: p= 0.013 and LC_{90}: p= 0.008) compared to control series. Moreover, the treatment had no significant effect ($p>0.05$) on SSCC of carbohydrates and lipids as compared to control values.

Table 7: Effect of spiromesifen (LC_{50} and LC_{90}) on metabolites specific size caloric indexes (SSCC) (Cal/mm^3) on fourth instar larvae of *C. pipiens* (mean ± SD, n= 3 pools each containing 10 individuals). In each line, values followed by the same letter are not significantly different at $P>0.05$.

Treatment	Control	LC_{50}	LC_{90}
Protein SSCC	0.04 ± 0.00 a	0.07 ± 0.00 b	0.09 ± 0.00 c
Carbohydrate SSCC	0.10 ± 0.01 a	0.09 ± 0.01 a	0.09 ± 0.00 a
Lipid SSCC	0.17 ± 0.01 a	0.15 ± 0.01 a	0.12 ± 0.01 a

Discussion

Insecticidal activity

The intensive use of synthetic pesticides have caused secondary effects on the environment [35].The application of spiromesifen and buprofezin on *Bemisia tabaci* and *Bemisia argentifolii* causes a reduction in the number of population particularly in the nymphal stage [36, 37]. In addition, the same product used at different doses (0.0024; 0.024; 0.24; 2.4 and 24 mg/L) exhibited an insecticidal activity on *Bactericera cockerelli* (Hemiptera: Triozidae) nymphs [38]. In the current study, spiromesifen tested on fourth-larval instar of *Culex pipiens* was found to present an LC_{50} of 542.21 µg/L and an LC_{90} of 1148.65µg/L. The study of Djeghader *et al.* (2013)[8] conducted in *Culex pipiens* using novaluron, an inhibitor of chitin synthesis showed the following values LC50 and LC90 were 0.32 and 1.2 µg/l for the third-instar larvae, while these respective values were 0.58 and 2.2 µg/l for the fourth-instar larvae, respectively. In addition, the chitin synthesis inhibitors appeared more potent against *Culex pipiens* larvae as compared to molting hormone agonists, juvenile hormone analogues or spiromesifen (Table 8).

Table 8: Efficacy of several insecticides against fourth instar larvae of *C. pipiens.*

Insecticides	LC_{50}	References
Teflubenzuron	5.92 ng/L	(39)
Diflubenzuron	16.36 ng/L	(39)
Flucycloxuron	35.81 ng/L	(6)
Triflumuron	36.25 ng/L	(40)
Novaluron	0.58 µg/L	(8)
Halofenozide	12.58 µg/L	(9)
Pyriproxyfen	0.84 µg/L	(41)
Kinoprene	246.8 µg/L	(42)
Spiromesifen	542.21 µg/L	Current study

Effect on biomarkers

To contribute to an understanding of these mechanisms, we assessed the effect of the spiromesifen on the activity of a biomarker of oxidative stress (MDA, CAT) in *C. pipiens*. The results show a significant increase in the rate of MDA following exposure to spiromesifen (LC_{50} and LC_{90}) compared to controls confirming our previous experiment made in *Drosophila melanogaster* [13]. Spiromesifen treatment also induced an incresae in the CAT activity in the fourth instar larvae of *C. pipiens*. This increase in activity reflects an establishment of the process of detoxification, which is a form of defense of the insect against the pesticide [43].

Effect on growth

The body size is an important trait for mosquitoes, because its influence on the blood-feeding ability, host attack rate and fecundity. All of these traits are determinants of their potential to transmit diseases [44]. In the present study the application of spiromesifen (LC_{50} and LC_{90}) induced a significant decrease in the weight and the volume of larva body of the fourth stage in *C. pipiens* compared to controls. Novaluron was also found to reduce significantly of the body weight in third and fourth larval stage of *Culiseta longiareolata* [45].

Effect on biochemical composition

In insects, the hemolymph undergoes metabolic modification during the developmental stages [46, 47]. The exposure of an organism to xenobiotic products can modify the synthesis of certain metabolite and disturb its functionality [48]. Biochemical analyzes (carbohydrates, lipids and proteins) revealed a decrease in the levels of lipids and proteins in whole body extracts in spiromesifen treated larvae as compared to control series. The reduction in protein levels observed in *C. pipiens* larvae might be due to their degradation for metabolic ways or to an impaired incorporation of amino acids into polypeptide chains or inhibition of protein synthesis. As reported by Ghasemi *et al.* (2010) [49], pyriproxyfen caused a significant decrease in protein contents in *Plodia interpunctella* (Lepidoptera). Similarly, treatment of *Bemisia tabaci* with spiromesifen was found to affect the lipid contents [37]. The effects of spirodiclofen and hexaflumuron were also investigated on some physiological changes of the last instar larvae of

Hippodamia variegate by measuring total lipid contents [50]. Our results also showed a significant reduction in carbohydrate levels only with the highest dose. Ali Mohamadi *et al.* (2014) [50] reported a reduction in glycogen contents in fourth instar larvae of *H. variegate* after treatment with hexaflumuron and spirodiclofen.

Caloric indexes

The ability of a mosquito to survive, and therefore to transmit disease depends largely on its caloric reserves [51]. Moreover, the blood meal volume and the number of mature eggs produced are influenced by the body volume of mosquito females [52]. In the present study, treatment with sipromesifen (LC_{50} and LC_{90}) affected the caloric content of the major constituents (proteins, carbohydrates and lipids) of the larvae *C. pipiens*. Tine-Djebbar & Soltani (2008) [34] have also shown that halofenozide (RH-0345), an insect growth regulator mimetic of the molting hormone affected the caloric indexes in *Culiseta longiareolata*.

Conclusion

This study was conducted to offer a preliminary understanding of the role played by spiromesifen against *C. pipiens*. The results obtained using spiromesifen applied at two lethal concentrations (LC_{50} and LC_{90}) on fourth instar larvae showed that treatment disrupt the biochemical composition, as well different morphometric measurements. Moreover, it appears less potent than other insecticides tested such as the insect growth disruptors.

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