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Spiromesifen, an insecticide inhibitor of lipid synthesis, affects the amounts of carbohydrates, glycogen and the activity of lactate dehydrogenase in *Drosophila melanogaster*

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Abstract
Spiromesifen (Oberon® 240 SC), an insecticide/acaricide widely used to control pests like mites and whiteflies, was applied topically at two sublethal concentrations (LC_{10}: 21.45 µg/µl and LC_{25}: 39.53 µg/µl), on newly molted pupae of *Drosophila melanogaster* Meigen, 1830 (Diptera, Drosophilidae). The effects of treatment were evaluated on the duration of pupal stage, changes in the pupal weight, the amounts of carbohydrates and glycogen and the rate of the lactate dehydrogenase. Results show that spiromesifen reduces the amounts of both total carbohydrates and glycogen in pupae of *D. melanogaster* as compared to controls. Moreover, treatment also caused a significant increase in the lactate dehydrogenase activity. The biochemical measurements of carbohydrates and glycogen confirm that *D. melanogaster* exhibit a U-shaped metabolic curve and spiromesifen treatment disturbs the energy metabolism and induces a chemical stress resulting in an increase in LDH activity.

Keywords: Energy metabolism; Spiromesifen; *Drosophila melanogaster*; Carbohydrates; Glycogen; Lactate dehydrogenase.

1. Introduction
The crop protection industry has developed more selective molecules that act on specific biochemical processes in target organisms [1]. Spiromesifen is a systemic product belonging to the chemical group of spirocyclic tetronic/tetramic acid derivatives widely used against certain pests like mites and whiteflies [2]. This insecticide/acaricide has become an important element in the resistance management program [3] due to its mode of action and low toxicity against no target organisms [4]. It acts on lipid synthesis by inhibiting acetyl CoA carboxylase [5].
During the life of an insect, energy-requiring processes such as molting, growth, vitellogenesis, muscular activity, and fasting lead to increased metabolism. Fat body cells not only control the synthesis and utilization of energy reserves but also synthesize most of the hemolymph proteins and circulating metabolites [6]. Carbohydrates are an important source of energy and modulate many vital metabolic and developmental processes [7, 8]. Glycogen and triglyceride are the energy reserves in animal cells. Glucose is stored in a polymeric form, glycogen that can be readily degraded on demand to be used as a glycolytic fuel [9]. Recently, *Drosophila melanogaster* Meigen, 1830 (Diptera, Drosophilidae) has been used as a target model to investigate the impact of selective insecticide on reproduction and development [10, 11, 12]. In this species the primary fuel used during metamorphosis was lipid, which accounted for more 80% of total metabolism [13] and spiromesifen was reported to affect the amounts of body lipids and the rate of malondialdehyde [10]. The metabolism of carbohydrates has been intensively reviewed [14, 15, 16]. Lactate dehydrogenase (LDH) (EC 1.1.1.28) an important glycolytic enzyme [17] is involved in carbohydrate metabolism and has been used as biomarker of chemical stress [18, 19, 20]. In addition, LDH is a parameter widely used in toxicology and in clinical chemistry to diagnose cell, tissue and organ damage. However, their potential use as a biomarker of environmental stress in invertebrate organisms has been scarcely explored [21]. Therefore, in our current study we used *D. melanogaster* as an experimental model. Spiromesifen was applied topically to pupae at two sublethal concentrations (LC_{10} and LC_{25}) and their effects examined on the rate of carbohydrate and glycogen, and the specific activity of lactate dehydrogenase.

2. Materials and methods

2.1 Fly rearing and maintenance

Drosophila melanogaster Meigen 1830 strain Canton S was obtained from Dr. J.P. Farine (CSGA, CNRS UMR 6265, Faculty of Sciences, University of Bourgogne, France) and reared on plastic vials containing an artificial medium consisting of corn meal, agar, yeast, and methyl-4-hydroxy-benzoate and maintained at 25±2 °C and a relative humidity of 65±5% under a photoperiod of 12 h light: 12 h dark as previously described [12].

2.2 Insecticide and treatment

Spiromesifen (Oberon® 240 SC: concentrated solution; Bayer Crop Science, Germany) was obtained from Bayer Crop Science (Algeria). The compound was applied topically to newly molted pupae at two sublethal concentrations (LC_{10}: 21.45 g /insect and LC_{25}: 39.53 g /insect), previously determined [10]. Controls were treated with the solvent alone (1µl acetone/distilled water; 50/50).

2.3 Carbohydrates, glycogen and protein extraction

Carbohydrates, glycogen and proteins were extracted from the same sample according to the procedure of Shibko *et al.* (1966) [22]. In brief, each pooled sample consisting of 4 pupae collected at various time during the pupal stage was homogenized (Sonifier B-30) in 1 ml of trichloroacetic acid (20%) and centrifuged (5000 x g for 10 min). The supernatant-1 was used for carbohydrate and glycogen quantification while the precipitate was washed with 1 ml of ether-chloroform (1:1; v:v) and centrifuged as described above to obtain the precipitate-2 that was suspended in 1 ml of NaOH (0.1N) and used for total protein quantification.

2.4 Carbohydrate, glycogen and protein quantification

The quantification of the amounts of total carbohydrates was performed using the anthrone–sulfuric acid method [23]. It consists to add 100 µl of the supernatant to 4 ml of the anthrone reagent and the homogenate was incubated at 80 °C for 10 min. The absorbance was measured at 620 nm. Glycogen was assayed following the procedure of Van Handel (1985) [24]. Briefly, this method consists in adding to the supernatant one (containing glycogen) 50 µl of sodium sulfate saturated solution in water plus two drops of absolute ethanol. After centrifugation (5000 g, 10 min at 4 °C), the precipitate was recovered and added to 1 ml of Na-saturated solution in ethanol 66%. A second centrifugation was performed and then 2 ml of the anthrone reagent was added to the precipitate. The homogenate was incubated at 100 °C for 13 min. The absorbance was read at 620 nm. Glycogen (Sigma-Aldrich, USA) was used as a standard. Protein content was measured by the Coomassie Brilliant Blue G-250 dye-binding method [25] with bovine serum albumin as a standard. The absorbance was measured at 595 nm.

2.5 Lactate dehydrogenase assay

Newly molted pupae of *D. melanogaster* were treated topically with spiromesifen at its LC_{10} and LC_{25}. Samples were collected at different times (0, 24, 48 and 72 h) following treatment. The enzyme assay was assessed following the procedure of Hill & Levi (1954) [26] as previously described [27]. Briefly, each pooled sample (4 pupae) was homogenized in 1 ml buffer Tris/HCl (0.1M; pH 7.2). After centrifugation (10.000 g/min for 5 min), the supernatant was recovered and used as an enzyme source. The assay was performed with 50 µl of supernatant added to 675 µl of substrate buffer (0.2 M; pH 10) and 50 µl of coenzyme NAD solution. The absorbance was read every minute for 5 minutes at 340 nm. The specific activity of LDH was expressed in µmol/ min/ mg proteins) and determined by the formula of Audigie *et al.* (1982) [28].

2.6 Statistics

Data are presented as means ± standard deviation (SD) and subjected to analyses of variance (ANOVA) followed by Turkey's test. All analyzes were performed using MINITAB (version 16, PA, State College, USA) and $p<0.05$ was considered statistically different. The number of pupae tested is given with the results.

3. Results

3.1 Effects of spiromesifen on growth and development

The effect spiromesifen applied topically at two doses (LC_{10} and LC_{25}) on newly ecdysed pupae was examined on the duration of pupal stage and the weight of pupae. Treatment had no significant effect ($p>0.05$) on the duration of the pupal stage as compared to control group (Control= 4.27±0.07 days; $LC_{10=}$ 4.26±0.07 days; $LC_{25=}$ 4.29±0.04 days). Changes in weight of pupae are presented in Table 1. They show that the profile of weight changes during the pupal stage was similar in control and treated series and characterized by a slight decrease in the weight during the pupal development. In addition, the compound caused a significant reduction ($p<0.001$) in the weight of pupae only at day 1 with the highest dose as compared to controls (Table 1). The analysis of variance indicated significant effects of age ($F_{3.\ 48} = 327.30$; $p<0.001$), treatment ($F_{2.\ 48} = 16.64$; $p<0.001$) and age/treatment interaction ($F_{6.\ 48} = 4.95$; $p = 0.001$).

Table 1: Body weight (mg) of *D. melanogaster* pupae during the pupal stage (Mean±SD; n= 5 repeats each corresponding to the weight of a pupa, for the same treatment, means followed by the same letter in small are not significantly different, while for the same age, means followed by the same letter in capital are not significantly different at $p> 0.05$).

Treatment	Age (Days)			
	0	1	2	3
Control	1.54±0.03 a A	1.41±0.04 b A	1.26±0.02 cd A	1.18±0.04 d A
Spiromesifen (LC_{10})	1.53±0.02 a A	1.33±0.05 bc AB	1.23±0.02 c A	1.19±0.01 d A
Spiromesifen (LC_{25})	1.49±0.04 a A	1.29±0.02 bd B	1.22±0.01 cd A	1.17±0.05 d A

3.2 Effects of spiromesifen on carbohydrate and glycogen amounts

Measurements of total carbohydrates and glycogen in whole body extracts of pupae were presented in Tables 2 and 3. Results show that these during metamorphosis are similar for both the two biochemical components in control and treated series. Indeed, changes followed a U-shaped pattern characterized by a decrease after pupation (at day 1 and 2) and

an increase (at day 3) shortly before the emergence of adults stage. When applied topically to newly molted pupae spiromesifen at the two tested doses resulted in a significant (p>0.05) decrease in carbohydrate amounts at days 1, 2 and 3 following treatment as compared with control groups. However, there was no significant (p>0.05) difference between the two tested doses. The analysis of variance revealed significant effects of age ($F_{3.\ 48}$ = 980.42; p<0.001) and treatment ($F_{2.\ 48}$ = 2.38; p= 0.077) and age /treatment interaction ($F_{6.\ 48}$ = 30.92; p<0.001).

Table 2: Body carbohydrate contents (µg/mg of fresh tissue) in control and treated series during the pupal development of *D. melanogaster* (m±SD; n = 5 repeats each corresponding to a pool of 4 pupae; for the same treatment, means followed by the same letter in small are not significantly different, while for each age values followed by the same letter in capital are not significantly different at p> 0.05).

Treatment	Age (Days)			
	0	1	2	3
Control	164.18±3.17 a A	116.12±3.54 b A	116.74±3.61 b A	146.20± 2.24 c A
Spiromesifen (LC_{10})	162.20±3.19 a A	108.15±1.58 b B	133.01±2.24 b B	134.54±3.16 b B
Spiromesifen (LC_{25})	164.70±3.11 a A	107.98±1.62 b B	129.44±2.92 c B	133.75±2.55 c B

The evaluation of glycogen amounts presents a significant decrease at day 1, 2 and 3 only with the highest dose (LC_{25}) in comparison to controls (Table 3). Two-way analysis of variance (ANOVA) reveals significant effects of treatment ($F_{2.\ 48}$= 157.02; p<0.001) and age ($F_{3.\ 48}$= 30.17; p<0.001). However, the interaction (age / treatment) was not significant ($F_{6.\ 48}$= 0.81; p > 0.05).

Table 3: Body glycogen contents (µg/mg of fresh tissue) in control and treated series during the pupal development of *D. melanogaster* (m±SD; n = 5 repeats each corresponding to a pool of 4 pupae; for the same treatment, means followed by the same letter in small are not significantly different, while for each age values followed by the same letter in capital are not significantly different at p> 0.05).

Treatment	Age (days)			
	0	1	2	3
Control	100.11±2.02 a A	80.29±1.68 b A	59.73±2.22 c A	67.57±2.57 d A
Spiromesifen (LC_{10})	96.86±2.13 a A	76.37±3,18 b AB	53.84±1.07 c AB	63.65±1.07 d AB
Spiromesifen (LC_{25})	97.90±4.07 a A	70.49±2.11 b B	47.96±2.12 c B	57.76±2.12 d B

3.3 Effects of spiromesifen on LDH activity

The specific activity of lactate dehydrogenase (LDH) was estimated in the control and treated series by applying the formula of Audigie *et al.,* (1982) [28] using the slopes of the regression lines, expressing the absorbance versus time. In control series, the specific activity of LDH presents a peak at 2 days. The specific activity of LDH in spiromesifen treated series shows a similar trend to that of controls but with a significant increase (p<0.001) at all ages following treatment. In addition, a dose-dependent effect was observed. Two-way ANOVA reveals significant effects of treatment ($F_{2.\ 48}$= 8041.63; P<0.001), age ($F_{3.\ 48}$= 48603.57; P<0.001) and interaction age / treatment ($F_{6.\ 48}$= 1652.49; P<0.001).

Table 4: Changes in the specific activity of lactate dehydrogenase (µM/ min/ mg of proteins) in control and treated series during the pupal development of *D. melanogaster* (m±SD; n = 5 repeats each corresponding to a pool of 4 pupae; for the same treatment, means followed by the same letter in small are not significantly different, while for each age values followed by the same letter in capital are not significantly different at p> 0.05).

Treatment	Age (days)			
	0	1	2	3
Control	25.64±0.10 a A	38.19±0.40 b A	62.22±0.44 c A	32.01±0.21 d A
Spiromesifen (LC_{10})	23.93±0.14 a AB	59.83±0,21 b AB	74.96±0.45 c AB	43.54±0.19 d AB
Spiromesifen (LC_{25})	25.63±0.27 a B	61.41±0.21 b B	77.47±0.35 c B	54.16±0.11 d B

4. Discussion

In the current study, spiromesifen applied topically on newly molted pupae of *D. melanogaster* had no significant effect on the duration of pupal stage but reduced the body weight of pupae only with the highest concentration at day 1 following treatment. Similarly this compound does not interfere with the duration of development in *Bemisia tabaci* biotype B and has moderate effects on adult and nymphal stages [29, 2]. Mahmoudvand *et al.* (2011) [30] reported in *Plutella xylostella* a decrease in body weight of pupae treated with sublethal concentrations of indoxacarb, while Soltani-Mazouni *et al.* (2012) [31] observed in *Ephestia kuehniella* a reduction in the duration of the pupal stage with a significant dose-response following application of methoxyfenozide a molting hormone agonist.

Metamorphosis represents a critical period in which energy stores established from larval feeding are allocated between fueling pupal development and supporting the needs of the adult development and reproduction [13]. Thus, the metamorphic changes are usually accompanied by substantial depletion of carbohydrate reserves. The metabolism of carbohydrates has been intensively reviewed [14, 15, 16], and the fat body plays a major role in intermediary metabolism and it is the central storage depot of nutrients and energy reserves and responds to energy demands liberating both glucose and trehalose to the hemolymph [6, 8].

In the present experiment, both total carbohydrates and glycogen exhibit a similar profile during the metamorphosis of *D. melanogaster*. This pattern characterized by a decrease after pupation and an increase prior to adult emergence was demonstrated in several insect orders including Diptera [13]. The decrease observed was more marked after topical application of spiromesifen. It coincides with the peak of ecdysteroids and the initiation of the adult cuticle [32], and could be explained by a glucose mobilization which provides an energy source and a substrate for synthesis of adult cuticle [15], and also a response to stress induced by chemical treatment [33].

Similarly, a decrease in glycogen in the presence of a toxic stress in *Gammarus roeseli* [34] or in *Lymnaea acuminata* [35]. However, if Choi *et al.* (2001) [36] observed in *Chironomus riparius* a decrease in glycogen content after 24 hours of exposure to tebufenozide, they also note that content finds its initial level after 48 hours of exposure. Rivero *et al.* (2007) [37] observed a decrease in glycogen and lipid levels in the mosquito *Aedes aegypti* larvae after treatment with

diflubenzuron. In contrast, results were observed in dipteran exposed to spinetoram which leads to increased glycogen reserves that could be related to impaired catabolism of glycogen [38].

Changes in specific activity of LDH exhibit a similar trend in control and spiromesifen treated series but with a significant increase in all ages following treatment. LDH is involved in the production of energy, being particularly important when a considerable amount of additional energy is required immediately [39]. A stimulation of LDH suggests that the final product of glycolysis, pyruvate, is preferably used for the production of lactate in order to support the large and rapid energy demand caused by chemical stress [40]. An induction in LDH activities was also observed in *Blattella germanica* treated by several conventional insecticides [41, 42, 43] or in *Tribolium castaneum* treated with Dimilin [44]. A highest activity of LDH was observed in the larvae of *Chilo suppressalis* exposed to hexaflumuron [45].

5. Conclusion

The biochemical measurements performed in *D. melanogaster* suggested an increased energy demands in spiromesifen treated pupae and also possible damages to pupal body since LDH is a biomarker of tissue damage in animals [18, 19, 20].

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