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Evaluation of the Potential Side-Effects of Novaluron on the Shrimp *Palaemon adspersus*: Moulting Hormone Profile, Cuticle Secretion and Chitin Contents

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Abstract: The leaching of a large amount of pollutants derived from agricultural and domestic activities (fertilizers, pesticides, detergents) might contaminate especially the aquatic environments affecting several non-target aquatic organisms such as crustacean species. The current study aimed to evaluate under laboratory conditions the potential side-effects of novaluron (20% Wettable Powder), a potent benzoylurea derivative insecticide on mosquito larvae, against a non-target shrimp, *Palaemon adspersus* Rathke, 1837 (Decapoda, Palaemonidae). This species is abundant in the lagoon El-Mellah (Northeast Algeria) and a relatively important species for the local fishery industry. The compound was tested at two concentrations (0.91 mg/L and 4.30 mg/L) corresponding respectively to the LC_{50} and LC_{90} determined against fourth-instar larvae of *Culiseta longiareolata* (Diptera, Culicidae). The newly ecdysed adult shrimps were exposed for 15 days, i.e. stage A until D during a moult cycle. Under normal conditions, changes in hemolymphatic ecdysteroid concentrations during the molting cycle presented a peak at stage D, just before the ecdysis while in the treated series, we note an increase in hemolymphatic ecdysteroid concentrations at stages C and D and an absence of the peak as compared to the controls. Histological observations of integuments revealed that novaluron caused a significant reduction in thickness of the new cuticle at its LC_{50} and an inhibition of the new cuticle secretion at its LC_{50}. The determination of chitin amounts, showed that exposure of shrimps to novaluron resulted in a significant decrease of values at all molting stages with a dose-response manner in comparison to controls. Thus, the overall data confirm the primary mode of action of novaluron on chitin. This insecticide can present secondary effects on this non-target shrimp species commercially important for the local economy.

Keywords: Toxicology, Novaluron, *Palaemon adspersus*, Ecdysteroids, Cuticle, Chitin

1. Introduction

Conventional pesticides are widely used in crop production and very effective against target organisms [1]. So, they are known to make risks and impacts on human health and environment [2]. In this context, several institutions have extensively searched alternatives such as insect growth disruptors (IGDs) with specific mode of action on insect and lower toxicity against non-target organisms than conventional insecticides [3, 4]. The IGDs compounds can be grouped according to their mode of action, as follows: substances that interfere with the action of insect hormones (i.e. juvenile hormones, ecdysteroids) and chitin synthesis inhibitors (i.e. of cuticle formation. Among these they are several classes of the chitin synthesis inhibitors, such as pyrimidine-nucleoside peptides, benzoylurea, oxazolines, thiazolidines, tetrazines, thiadiazines, thiophthalimides and certain chromo- and fluorophores [5]. The benzoylurea compounds prevent the formation of chitinous structures and interfere with the molt process which hampers normal development of exoskeleton in many insect orders [6]. During the last decades, an intensive search for more potent benzoylurea derivatives from the prototype compound, diflubenzuron [7], has resulted in synthesis of several

analogues, such as triflumuron [8], chlorfluazuron [9], teflubenzuron [10], hexaflumuron [11], flufenoxuron [12], lufenuron [13] and more recently, novaluron [14]. Previously, it has been shown that diflubenzuron could affect the cuticle by reducing the thickness and altering their structure [15] due to a decreased amount of chitin in *Penaeus kerathurus* [16]. An HPLC analysis for residues of diflubenzuron, and has reported that the compound present a low stability in sea water under laboratory conditions [15]. More recently, diflubenzuron affect the levels of different biochemical constituents as proteins, lipids, carbohydrates in hemolymph and muscle during a moulting cycle [17].

Novaluron is a chitin synthesis inhibitor, belonging to the class of benzoylurea insecticide with excellent activity against several important insect pests [18] with a high toxicity level and effectiveness against several mosquito larvae as, *Culiseta longiareolata* [19] *Aedes aegypti* [20] and *Culex pipiens* [21]. It was designated a reduced-risk/organophosphorus alternative as it exhibit low acute mammalian toxicity and no significant subchronic effects in mammals [22, 23, 24]. So, according to these agencies, novaluron was considered a low risk to the environment and non target organisms. Its use might contaminate rivers which diverse their pollutants into the lakes of El kala (Northeast Algeria) and the Annaba gulf. Therefore, in the present study, we investigate the impact of this compound on a non-target organism, shrimp *Palaemon adspersus* Rathke, 1837 (Decapoda, Palaemonidae) abundant in the lagoon El-Mellah (Northeast Algeria) and a relatively important species for the local fishery industry. The compound was added to the rearing seawater of newly-ecdysed adult shrimps during a molt cycle. We examine its effects on ecdysteroid profile, cuticle secretion and chitin contents. The data obtained show that this insecticide can present secondary effects on this non-target shrimp species.

2. Materials and Methods

2.1. Collection and Rearing of Shrimps

Palaemon adspersus Rathke, 1837 (Decapoda, Palaemonidae) were collected from the lagoon El-Mellah (Northeast Algeria), in the channel that leads to the Mediterranean Sea (Figure 1). This site is far from any source of pollution and expected as a relatively clean site away from pollution sources [25, 26]. Shrimps were transported to the laboratory alive and reared in laboratory conditions by maintaining them in glass aquaria (100 x 60 x 80 cm) filled with sea water (salinity 37 psu; temperature 22-25°C; photoperiod 14 h of light). Filtration is performed by water filter having a flow rate of 180 1 / h (Rena 225). The animals were daily fed with fresh mussels distributed in the afternoon. Prior to exposure, shrimps were acclimated to laboratory conditions for a week. Shrimps with of similar size (length: 25 mm and weight: 850 mg) were used in the experiment.

Figure 1. *Location of the sampling site in the Mellah lagoon (Northeast Algeria). [27] *: constriction zone of the channel with Mediterranea.*

2.2. Shrimp Datation

The decapod Crustaceans moult cycle is divided into five key stages: A (early postmolt), B (late postmolt), C (intermolt) and D (premolt) and moulting (E). The datation was made according to the method of [28], based on morphogenesis be at the uropod. This method is simple, fast and efficient. Under these conditions, *P. adspersus* has a molt cycle of 20 days with 20% for A+B, 25% for C, and 65% for D.

2.3. Insecticide and Treatment

Novaluron (wettable powder 20% active ingredient), was kindly provided by Pr. G. Smagghe (Ghent University, Belgium) (Figure 2). The compound was added to the rearing sea water at two final concentrations (0.91 µg/L and 4.30 mg/L) corresponding respectively to the LC_{50} and LC_{90} obtained with respect to the fourth-stage larvae *Culiseta longiareolata* (Diptera, Culicidae) [19]. Newly-ecdysed adult shrimps (0-8 h old) were exposed continuously to treatment. Control shrimps were reared in sea water only. Samples (hemolymph, cephalothorax and uropod) were collected from each shrimp at different stages of molt cycle (A, B, C, and D) in control and treated series.

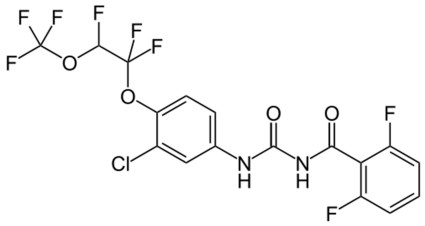

Figure 2. *Molecular structure of novaluron.*

2.4. Enzyme Immunoassay of Ecdysteroids

Each sample of hemolymph (3 µl) was extracted with methanol by sonication (2-3 min). After centrifugation (5000 g, 10 min), the supernatants were taken and evaporated (60°C). Each sample was resuspended in 500 µl of phosphate buffer (0.1M; pH7.4) and individually analyzed by an enzyme-immunoassay (EIA) according to the method of [29] modified by [30] and previously described [31] using a conjugate of 20-hydroxyecdysone coupled to peroxidase as the enzymatic tracer, tetramethylbenzidine as the colour reagent and a rabbit B polyclonal antibody. Absorbance was read at 630 nm and data was expressed in pg 20E/µl of hemolymph. The tracer and antibodies were kindly provided by Dr. J. P. Delbecque (CNRS, University of Bordeaux I, France) and C. Blaise (Pierre and Marie Curie University, Paris, France), respectively.

2.5. Histological Procedure

Uropods were sampled at different stages of moult cycle (A–D), in control and novaluron-exposed series and fixed in formol (10%). After dehydration in serial washes of graded ethanol the samples were passed through three washes in xylene before were embedded in paraffin as according to [32]. Transverse sections of uropod (4 µm) were made using a Leica RM2125T (Leica Microsystems Nussloch GmbH, Wetzlar, Germany) manual rotary microtome and stained with hematoxylin-eosin. Observations were made in a Leica DM500 microscope equipped with a Leica ICC50 HD camera and the thickness of different cuticle was measured with Las EZ Leica software in each series.

2.6. Chitin Quantification

Chitin quantification in peripheral integument was performed following the procedure of, previously described [33]. Chitin content was determined at different stages during the molting cycle in control and treated series by quantification of glucosamine derivatives obtained by deacetylation, depolymerisation and deamination of N-acetyl-glucosamine polymer. Briefly, chitin is subjected to an alkaline digestion with KOH (14 M) at 130°C to deacetylate the chitin of each sample, thus forming chitosan. Then a solubilized chitosan solution is depolymerized by $NaNO_2$ (10%) and $KHSO_4$ (10%) to liberate the amine residues from the glucosamine, forming a soluble aldehyde. Theses aldehydes generated in a reaction with $NH_4SO_3NH_2$ (12.5%) and with further addition of MBTH and Fe^{+3} a blue coloration. Absorbance was read at 650 nm and chitin content was expressed as glucosamine equivalents, according to a standard curve made with glucosamine. Weight of cuticle was determined, before chitin quantification to normalize the results.

2.7. Statistical Analysis

Statistical analyses were performed using the Prism software version 6.01 for Windows (GraphPad Software Inc., www.graphpad.com). Results are represented as mean ± standard deviation (SD). The homogeneity of variances was checked by Bartlett's test. The linear and non-linear regression was used to establish the reference curves for the determination of chitin and ecdysteroids contents, respectively. Data were subjected to two-way analysis of variance (ANOVA) followed by a post-hoc HSD Tukey test or to a Student's *t* test at $p< 0.05$.

3. Results

3.1. Effect of Novaluron on Ecdysteroid Contents

Under normal conditions of *P. adspersus,* the titers of hemolymphatic ecdysteroids increased during the molt cycle to reach a peak at stage D, just before the ecdysis. The value recorded at the beginning (stage A) and the end (stage D) were 33.48 ± 3.81 and 115.57 ± 2.51 pg/µl, respectively. In treated series by novaluron at the two tested concentrations (LC_{50} and LC_{90}), we note the absence of the peak of ecdysteroids at stage D and a significant increase ($p< 0.01$) at stages B, C and D as compared to control series. The values recorded with the LC_{90} were 93.76 ± 1.50 at the postmolt

(stage B), 119.63 ± 2.12 at the intermoult (stage C) and 137.44 ± 2.87 pg/µl at the premolt (stage D) (Table 1). In addition, ANOVA revealed significant effects of concentration ($F_{2, 24}$ =16.02; p< 0.0001), stage ($F_{3, 24}$ =124.8; p< 0.0001) and interaction concentration/stage ($F_{6, 24}$ = 2.06; p< 0.0001).

Table 1. *Effect of novaluron (LC_{50}, LC_{90}) on the hemolymphatic ecdysteroids titer (pg/µl equi 20E) during the molt cycle of P. adspersus (mean ± SD, n = 4-7).*

Stages	Control	Novaluron (LC_{50})	Novaluron (LC_{90})
A	33.48 ± 3.81 a A	36.41 ± 4.40 a A	30.57 ± 2.15 a A
B	68.57 ± 9.13 a B	90.84 ± 3.50 b B	93.76 ± 1.50 b B
C	83.77 ± 8.53 a B	116.59 ± 7.20 b C	119.63 ± 2.12 b C
D	115.57 ± 2.51 a C	139.23 ± 1.07 b C	137.44 ± 2.87 b C

Different capital letters indicate a significant difference between stages of the same series; different small letters indicate a significant difference between control and treated series of the same stage (p> 0.05).

3.2. Effect of Novaluron on Cuticle Secretion

In control series, the thickness of *P. adspersus* cuticle increased progressively during the three first stages (A, B, C) and decreased at the end of molt cycle (stage D) (Figure 3A). Cuticle thickness measurement showed that treatment with novaluron at the two tested concentrations (LC_{50}, LC_{90}), reduced significantly (p≤ 0.0001) the thickness of the old cuticle with a dose-response effect as compared to controls. ANOVA indicated significant effects of concentration ($F_{2, 28}$ = 35.82; p< 0.0001), stage ($F_{2, 28}$ = 47.3; p< 0.0001) and interaction concentration/stage ($F_{4, 28}$ = 4.37; p = 0.0035). As shown in figure 3B, the thickness of new cuticle was 3.31 ± 0.28 µm at stage D in control series. Novaluron-treatment decreased significantly (p≤ 0.0001) the thickness of this new cuticle (1.54 ± 0.48 µm) with LC_{50} and inhibited completely the secretion of the new cuticle with LC_{90}. The observations histological sections showed a reduction in the thickness of cuticles with both concentrations LC_{50} and LC_{90}without modifications in the structure appearance (Figure 4).

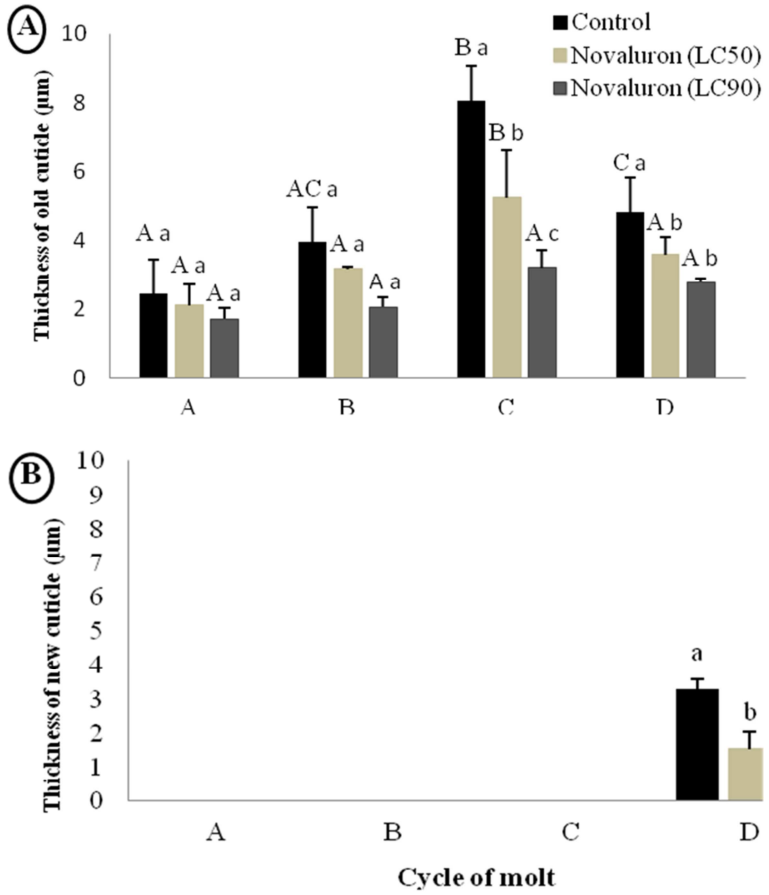

Figure 3. *Effect of novaluron (LC_{50}, LC_{90}) on the cuticle thickness measurement (µm) of old (A) and new cuticle (B) in P. adspersus during the molt cycle (m ± SD, n = 4-5).*

Different capital letters above values indicate a significant difference between stages of the same series; different small letters indicate a significant difference between control and treated series of the same stage (p> 0.05).

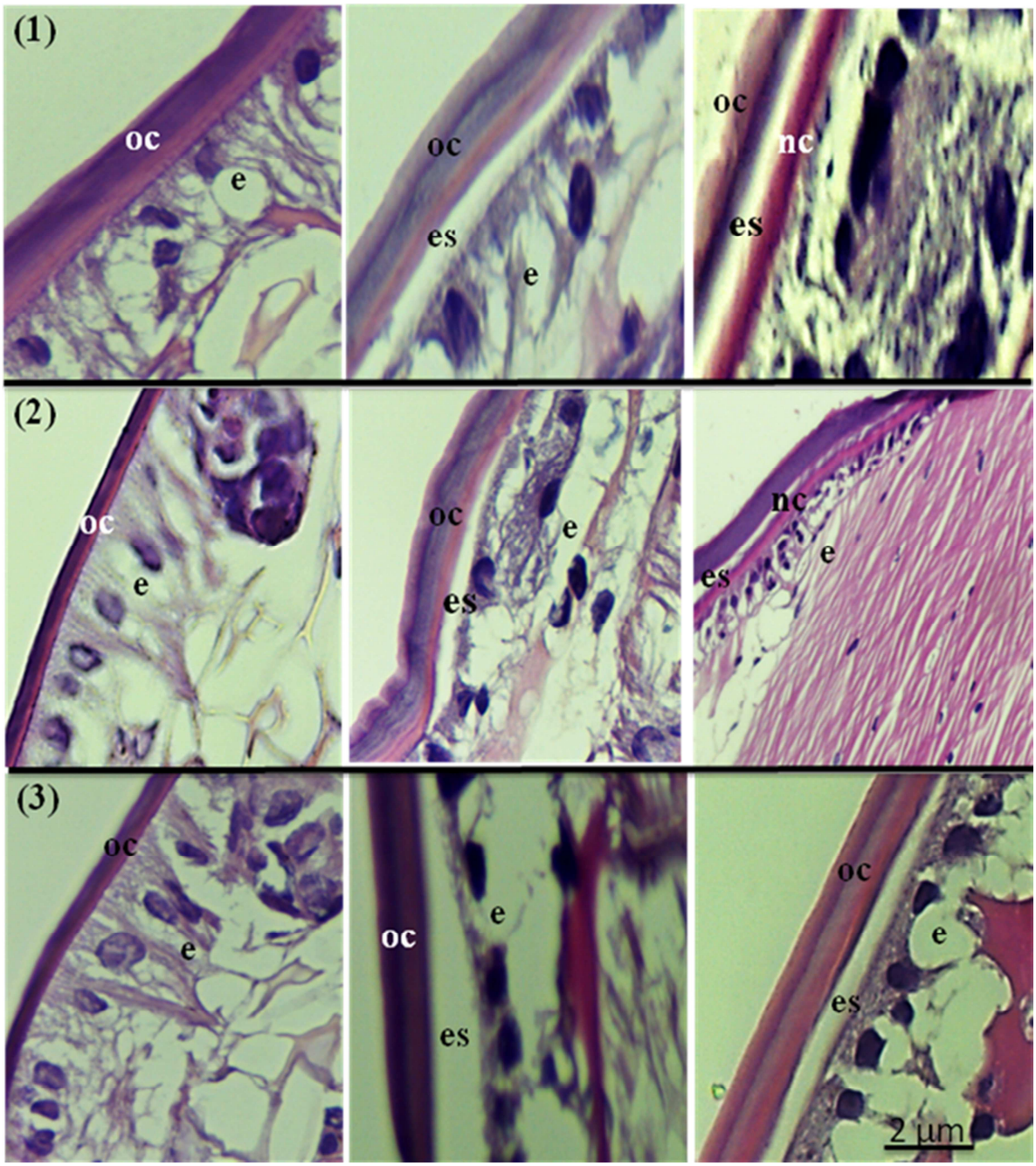

Figure 4. *Transverse sections of cuticle in control and treated series of P. adspersus during the molt cycle. (1) Control: stages A-B, C, D; (2) Novaluron LC_{50}: stages A-B, C, D; (3) Novaluron LC_{90}: stages A-B, C, D. (e: epidermis; oc: old cuticle; nc: new cuticle; es: exuvial space).*

3.3. Effect of Novaluron on Chitin Contents

The measurement of chitin contents in control series showed a progressive increase from stage A until stage C to reach a maximum of 150.37 ± 6.02 µg /mg and decreased thereafter at stage D (104.22 ± 8.45 µg/mg). Novaluron treatment (LC_{50} and LC_{90}), resulted in a significant ($p \leq 0.0001$) decrease in the chitin content with a dose-response relationship comparatively to controls. The values recorded with the LC_{50} were 97.82 ± 7.51 µg/mg at the stage C and 87.25 ± 8.88 at stage D. For, the LC_{90} the values decreased to 76.44 ± 4.63 µg/mg and 49.79 ± 4.38 µg/mg, at stages C and D, respectively (Table 2). ANOVA showed significant effects of concentration ($F_{2, 52} = 465.8$; $p< 0.0001$), stage ($F_{3, 52} = 216.4$; $p< 0.0001$) and interaction concentration/stage ($F_{6, 52} = 40.77$; $p< 0.0001$).

Table 2. *Effect of novaluron (LC_{50}, LC_{90}) on the chitin content (µg of glucosamine/mg tissue) during the molt cycle of P. adspersus (mean ± SD, n = 4-7).*

Stages	Control	Novaluron (LC_{50})	Novaluron (LC_{90})
A	71.12 ± 1.92 a A	73.03 ± 4.23 a A	48.51 ± 10 b A
B	101.71 ± 4.57 a B	85.43 ± 2.10 b B	67.06 ± 1.90 c B
C	150.37 ± 6.02 a C	97.82 ± 7.51 b C	76.44 ± 4.63 c C
D	104.22 ± 8.45 a B	87.25 ± 8.88 b B	49.79 ± 4.38 c A

Different capital letters indicate a significant difference between stages of the same series; different small letters indicate a significant difference between control and treated series of the same stage (p> 0.05).

4. Discussion

The molting hormone (ecdysteroids) in crustacean as in other arthropods, play a crucial role in the control of growth, reproduction and embryogenesis [34, 35]. The crustacean YO synthesizes from cholesterol as a precursor biosynthetic a greater diversity of ecdysteroids in the hemolymph [36, 37] depending on species such as, ecdysone, 3-dehydroecdysone (3dE), 25-deoxyecdysone (25dE) and 3-dehydro-25-deoxyecdysone (3D25dE). Peripheral tissues convert these compounds to the active hormone: 20-hydroxyecdysone (20E) and ponasterone A (25-deoxy-20-hydroxyecdysone or 25d20E by cytochrome P-450 mono-oxygenases [37]. 20E and PoA are the major active ecdysteroids circulating in the hemolymph during the postmolt and intermolt stages, while 20E alone is the major ecdysteroid during premolt stage of decapods crustacean [38, 39, 40]. Ecdysteroid assays were performed in a number of decapod crustaceans in total extracts, hemolymph, ovaries or eggs using a high performance liquid chromatography (HPLC) (Baldaia et al. 1984), the radioimmunoassay [40] and an enzyme-immunological method [31].

In the current study, under normal conditions, hemolymphatic ecdysteroid titers determined by an enzyme immunoassay, vary throughout the molt cycle of *P. adspersus*. The titers of 20E are low during postmolt (stage AB) and increased progressively in intermolt (stage C). A single peak was recorded in premolt (stage D). It coincides with the apolysis, which results from the destruction of the deep layers of the old cuticle and the beginning of the genesis of the new. In accordance with our results, total ecdysteroid titers in hemolymph vary over the molt cycle in a variety of crustacean species: *Orchestia cavimana* [40]; *Penaeus vannamei* [41]; *P. kearathurus* [31], and *Callinectes sapidu* [42]. Generally, in crustacean the titers of 20E is low during intermolt and postmolt; during premolt, concentration rise and reach a peak shortly before molting [43, 39].

The results obtained after treatment with novaluron, revealed an increase in hemolymphatic ecdysteroid titers with absence of the peak as compared to controls. The increase in hemolymph ecdysteroids is largely due to increased biosynthesis and conversion to active ecdysteroids. Novaluron is known to be very effective against several important insect pests [44, 45] and his bioactivity is typically much greater than diflubenzuron and teflubenzuron [14]. Indeed, different species of mosquitoes such as *A. aegypti* [46, 20]; *Culex spp* [47], *A. albopictus*, *Anopheles albimanus*, *Anopheles pseudopunctipennis* and *Culex quinquefasciatus* [48], *Culiseta longiareolata* [19] and *Culex pipiens* [21] were highly susceptible to novaluron. Several works demonstrated an increase in biochemical constituents (carbohydrates, proteins, lipids) with inhibitors of chitin synthesis: lufenuron in *schistocerca gregria* [49], novaluron in *Culex pipiens* [21] and flufenoxuron in *Schistocerca gregaria* [50]. Similar results were observed with shrimp's *P. kerathurus* after treatment with diflubenzuron; this compound caused an increase in the amounts of carbohydrates, lipids and proteins in the hemolymph at the end of the molting cycle (stage D) in *P. kerathurus* [17].

Histological study showed a progressive increase of the cuticle thickness during the three first stages (A, B, C) and a decrease at the end of the molt cycle (stage D) in controls. Cuticle thickness measurement showed that novaluron-treatment affect the cuticle secretion with a reduction in the thickness of the old cuticle with a dose-response manner as compared to control groups and an inhibition of the new cuticle. The application of chitin synthesis inhibitors typically induces malformations of the cuticle and a significant reduction of chitin amounts [51]. These results showed that the novaluron develop a fragile cuticle unable to support the increased tension during the molting process and the increase in chitin content observed during our experiments may be related to an inhibition of. Indeed, benzoylurea do not directly interfere with catalytic reaction of chitin synthesis, but act on a postcatalytic step [52], blocking the postcatalytic step of chitin synthesis [53]. Our results are consistent with those commonly reported. Indeed, the derivatives of the benzoylurea interfere with the molting process by disrupting cuticle secretion *via* the chitin synthesis [3, 15, 54]. Also, ultrastructural analysis revealed abnormal deposition of procuticular layers in response to the treatment with benzoylurea as demonstrated in shrimp *P. kerathurus* [15], beetles [51].

Chitin a polymer of *N*-acetyl-b-D-glucosamine, is a major component of the arthropods cuticle. It constitutes up to 40% of the exuvial dry mass depending on the species and varies considerably with the different cuticle types even in a single organism [55]. Chitin is catalyzed by the chitin synthase enzyme from UDP-N-acetylglucosamine precursors [52]. The molting hormone (20E) acts on expression and activity

of chitinolytic enzymes, such as chitobiase and chitinase which are involved in exoskeleton degradation and recycling during ecdysis in arthropods [56]. In our experiment, the measurement of chitin contents in controls showed a progressive increase from stage A until stage C and decreased at stage D. These variations were correlated with principal events of cuticle deposition. According to [15], the chitin content varied between 66 and 72% during molting cycle in shrimp *P. kerathurus*. The same authors reported an incorporation of two precursors, D-[3-^{3}H (N)]-glucose and N-acétyl-D-[1-3H]-glucosamine (NAGA) in the postmolt (stage A and B) leading to the secretion of endocuticle, followed by a decrease at the intermolt (stage C) (where the secretion of cuticle is complete) and the least content of incorporation of the two precursors is noted in premolt (stage D) where exocuticle secretion is completed. Novaluron-treatment increased significantly the chitin content with a dose-response effect probably by inhibit of the incorporation of sugars into the growing chitin chain**.** This is in accordance with a previous report made with diflubenzuron another chitin synthesis inhibitor on *P. kerathurus* [19].

5. Conclusion

In conclusion, the results obtained in this study were the first demonstrating that novaluron exerted negative effects in a shrimp species. It can increase the amounts of ecdysteroids and disrupt the chitin content causing inhibition of cuticular secretion in a non-target organism *P. adspersus.* These effects could be explained either by a blockage of transport and incorporation of the biosynthetic precursor of chitin, N-acetyl-D-glucosamine (GlcNAc), or directly by inhibition of chitin synthesis. However, these mechanisms of action remained unclear and new experimental approaches are needed. Given the biochemical composition of their cuticle, the crustaceans can be the potential targets of these benzoylurea derivatives.

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