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Full Length Research Paper

Enzyme immunoassay measurements of ecdysteroids in the last larval stage of *Culex pipiens* L. (Diptera, Culicidae): Hormonal profile and correlation with cuticle secretion

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The molting hormone (ecdysteroids) in whole body extracts of 4th-instar larvae of *Culex pipiens* L. (Diptera, Culicidae) was measured by an enzyme-immunoassay (EIA) using two specific antibodies, the rat monoclonal EC 19 antibody showing a high affinity for 20-hydroxyecdysone (20E) and the rabbit polyclonal B antibody six times more sensitive to ecdysone (E) than to 20E. EIA measurements revealed the presence of the two main hormones seen in *C. pipiens*: E (ca. 30 %) and 20E (ca. 70 %). Furthermore, change in ecdysteroid amounts during the 4th-larval stage was down using only a rat monoclonal because 20E was the major hormone. The ecdysteroid amounts presented a single peak that occurred at day three during the last larval development. In a second series of experiment, the cuticle cycle was determined by a histological study of the larval integument in order to establish temporal correlations with the hormonal levels. Thus, the ecdysteroid peak recorded during the last larval stage coincides with the apolysis and could be related with the induction of a new cuticle secretion.

Key words: Mosquitoes, *Culex pipiens,* hormone, ecdysteroids, enzyme-immunoassay (EIA), histology, cuticle.

INTRODUCTION

Mosquitoes are medically and veterinary important vectors, responsible for the transmission of many human and animal diseases, with more than one-half of the world's population at risk of malaria alone (Kaufman et al., 2011). *Culex pipiens* (Diptera, Culicidae) is the most widely distributed mosquito in the world and it carries a number of diseases (Lounibos, 2002). They are generally controlled by conventional insecticides (Cassida and Quistad, 1998). To reduce the impact of these conventional neurotoxins on the environment (Paoletti and Pimentel, 2000), more new selective chemicals are developed. Insect growth regulators (IGRs) seem promising. The IGRs such as molting hormone (ecdysteroids) agonists affect the hormonal regulation of molting and development processes (Dhadialla et al., 2005) by binding to the nuclear ecdysteroid receptor (EcR) as does the natural insect molting hormone (Dhadialla and Ross, 2007). Considerable progress has been achieved in understanding the blood meal digestion and utilization (Graf and Briegel, 1989), the effects of body size affecting reserve synthesis and fecundity (Briegel, 1990), and biology and ecology of mosquitoes (Clements, 1992). Due to their large geographical distribution, their abundance and their harmfulness, *C. pipiens* L. are the most interesting mosquito species in Algeria (Rehimi and Soltani, 1999; Tine-Djebbar and Soltani, 2008). The

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Abbreviations: EIA, Enzyme immunoassay; 20E, 20-hydroxyecdysone; **E,** ecdysone; **EcR,** ecdysteroid receptor.

normal development of insects and the regulation of the different physiological processes provide an experimental basis for investigating IGRs, which interfere with the insect's endocrine system, especially the molting hormone (ecdysteroids) (Dhadialla et al., 2005). The present study aimed at (for the first time) determining the nature of free ecdysteroids in whole body extracts and its changes during the 4th-larval stage of *C. pipiens* using an enzyme immunoassay. It is known that the molting hormone induces cuticle secretion (Lafont et al., 2005). Therefore, measurements of cuticle thickness during the 4th larval stage were made in order to establish temporal correlation with the hormone amounts.

MATERIALS AND METHODS

C. pipiens L. (Diptera, Culicidae) were obtained from a stock colony and kept as previously described (Rehimi and Soltani, 1999) at 25°C and a photoperiod of 14:10 (Light: Dark). 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.

Enzyme immunoassay for free ecdysteroids

We used in this study, whole body extracts because of difficulties to collect haemolymph samples from larvae as a result of their small size. Firstly, only free ecdysteroids were measured. The nature of free ecdysteroids in whole body extracts of larvae was shown using two specific antibodies kindly supplied by Dr. J. P. Delbecque (University of Bordeaux, France): a rat monoclonal EC 19 antibody showing high affinity for 20-hydroxyecdysone (20E) and a rabbit polyclonal B antibody six times more sensitive to ecdysone (E) than to 20E (De Reggi et al., 1992). Five newly molted 4th instar larvae (<8 h) were collected and each larval body was individually analyzed in duplicate by an enzyme immunoassay (EIA) as previously described (Soltani et al., 2002). Briefly, each sample was extracted with methanol by sonication, and after centrifugation (5000 *g*, 10 min), the supernatants were taken and evaporated. The extracts were resuspended in phosphate buffer (0.1 M, pH 7.4) and each sample was subjected to EIA using a conjugate of 20E coupled to peroxidase as the enzymatic tracer and tetramethyl benzidine as the colour reagent. Data obtained were corrected according to the antibody immuno-reactivity presented above. Furthermore change in ecdysteroid contents during the larval stage was determined using only a rat monoclonal antibody since 20E was the major hormone measured in larval body extracts. Thus, larvae were sampled daily during the larval stage and analysed individually as described above. Five larvae per time were used. The quantification of hormones was made by comparison with reference curves each established with serial concentration of E or 20E as standards. Results are expressed in picograms (pg) 20E equivalents per mg whole body.

Histology

In order to observe the cuticle secretion, larvae were sampled at various times during the 4th larval stage. Histological techniques were conducted according to Martoja and Martoja (1967). The abdomens were dissected and fixed in Bouin's solution. Finally, sections (5 to 7 µm) were stained with azocarmin-anilin and measurements were made on sternal cuticle from transverse sections of the abdomen. The thickness of both larval cuticle and the pre-ecdysial pupal cuticle was determined on five different larvae per time during the larval development and averaged.

Statistical analysis

Results are presented as the mean ± standard deviation (SD). The significance between different series was tested using student's *t* test at 5% level. All statistical analyses were performed using MINITAB Software (Version 13.31, Penn State College, PA, USA). The number of larvae tested in each experiment is given with the results.

RESULTS

EIA measurement of free ecdysteroids in larval body extracts

Samples from 4th larval stage of *C. pipiens* were used. Under the rearing conditions used, the duration (mean ± SD, n = 25 to 75) of 4th larval instar was 6.22 ± 0.69 days, while the mean body weight of individuals was 4.10 ± 0.82 mg (mean ± SD; n = 3 replicates each containing 100 individuals). The analysis of free ecdysteroids in newly molted 4th-larval stage of *C. pipiens* has been tested by an EIA. Data shows the presence of two main free ecdysteroid hormones E and 20E (Table 1) meanwhile, the antibody immuno-reactivity was corrected since rabbit polyclonal B antibody was 6 times more sensitive to E than to 20E (De Reggi et al., 1992). Thus, Table 2 shows that 20E, the active form of moulting hormone, was the major free hormone in the body extract of the larvae. It represents about 70% of the total free ecdysteroid detected in the larval body extracts (Table 2). Since 20E was the major free hormone in body extracts of *C. pipiens* larvae, quantitative changes in ecdysteroid amounts during the last larval stage was determined using a rat monoclonal EC 19 and data are expressed as pg 20E equivalent/mg wet body weight. The ecdysteroid EIA profile presented a single peak of 1.86 pg 20E equiv./mg body weight at day three (Figure 1) and the amount decreased to a basal level of 0.56 pg/mg at day six corresponding to the pupal ecdysis.

Cuticle secretion during 4th instar larval development

Histological study was made on abdominal sternal cuticles. The general view of abdomen transverse sections show the peripheral cuticle (Figure 2A), while the sternal integument from 6-day old 4th instar larvae indicates the secretion by the epidermis of a new cuticle under the old cuticle detached after the apolysis (Figure 2B). Measurements of sternal cuticles from 4th instar

Table 1. Contents (pg/larva) and amounts (pg/mg body fresh weigth) of ecdysone and 20-hydroxyecdysone in newly molted fourth-instar larvae of *C. pipiens* before the correction of the antibody immuno-reactivity (mean ± SD based on five replicates).

Hormone	Content (pg/larva)	Amount (pg/mg)
Ecdysone	1.58 ± 0.69	0.39 ± 0.02
20-Hydroxyecdysone	0.57 ± 0.13	0.14 ± 0.04

Table 2. Relative importance (%) of ecdysone and 20-hydroxyecdysone in newly molted fourth-instar larvae of *C. pipiens* after the correction of the antibody immuno-reactivity (mean ± SD based on five replicates).

Hormone	Importance (%)
Ecdysone	29.9 ± 6.2
20-hydroxyecdysone	70.1 ± 2.1

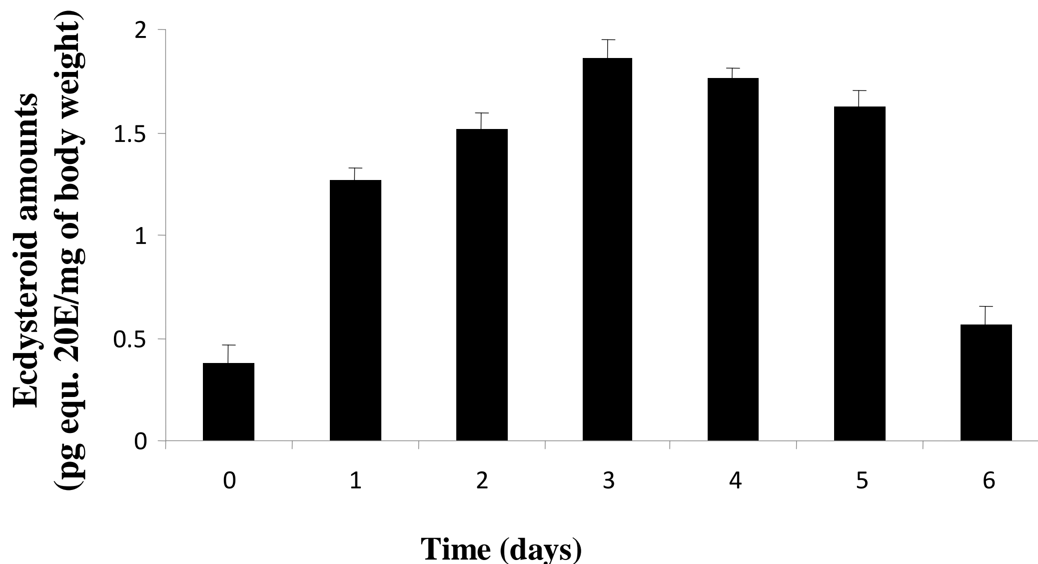

Figure 1. Changes in ecdysteroid amounts (pg equivalent 20E/mg of body weight) during the fourth-instar larval development of *C. pipiens* (mean ± SD based on five larvae per time).

larvae of *C. pipiens* showed that the thickness of larval cuticle, that is, the old cuticle, increased until apolysis (day three) to reach a maximum of about 3 µm and decreased thereafter (Figure 3). Underneath the old cuticle, deposition of the pre-ecdysial cuticle, that is, the new pupal cuticle, starts immediately after apolysis (day three). The thickness of the pre-ecdysial pupal cuticle, that is, the new cuticle, measured just before the pupal ecdysis (day six) was 1.44 ± 0.14 µm (Figure 3).

DISCUSSION

It is known that growth and development in insects are regulated by the steroid hormone and the sesquiterpenoid juvenile hormone (Nijhout, 1994). The prothoracic glands are the primary site of synthesis of ecdysteroids after their stimulation by a neurohormone secreted by the cephalic neurosecretory cells, the prothoracicotropic hormone (Gilbert et al., 2002). The first established function of ecdsyteroids was their role in the control of moulting processes (Lafont and Koolman, 2009). Hagedorn et al. (1975) demonstrated for the first time that mosquito ovaries are also a source of ecdysone. Later, the presence of ecdysteroids in ovaries was confirmed in several other insect species (Soltani-Mazouni et al., 1999). Ecdysteroids are synthesized by the follicle cells in the ovaries as an inactive form (ecdysone) and are involved in the regulation of repro-ductive maturation, and are also available to developing

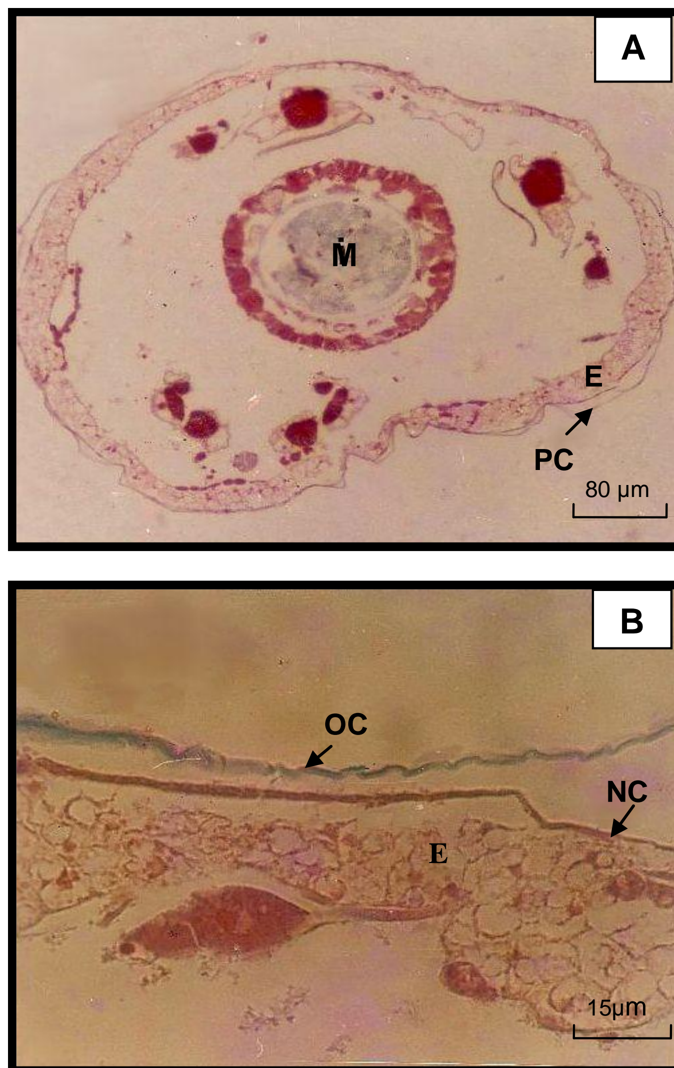

Figure 2. (A) Transverse sections of abdomen and (B) sternal integument of 6-day old fourth instar larvae of *C. pipiens* (fixation in Bouin's solution and staining with azocarmin-anilin). PC, Peripheral cuticle; M, midgut; E, epidermis; OC, old cuticle; NC, new cuticle.

embryos and pre-hatching larvae (Koolman, 1990; Gäde et al., 1997).

We presented here for the first time, data on moulting hormone in *C. pipiens* larvae. EIA measurements of free ecdysteroids made in whole body extracts of *C. pipiens* larvae revealed the presence of two main hormones; E and 20E. 20E, the active form of moulting hormone, was the major hormone. Purification of the ecdysteroids on thin layer chromatography and high performance liquid chromatography and analysis with radioimmunoassay indicate that in sugar-fed and blood-fed *Aedes aegypti* females, both E and 20E were present (Borovsky et al., 1986). This finding is in agreement with similar results for *Anopheles stephensi* (Redfern, 1982). Later, Birnbaum et al. (1984) demonstrated that both E and 20E are synthesized *in vitro* by *Aedes atropalpus* ovaries. However, earlier reports show that only 20E was identified from whole body extracts in *A. aegypti* (Hagedorn et al., 1975) and *A. atropalpus* (Masler et al., 1980). The predominance of 20E has also been reported in some species such as *Manduca sexta* (Bollenbacher et al., 1975), *Schistocerca gregaria* (Morgan and Poole, 1976), *Cydia pomonella* (Soltani, 1986), *Thaumetopoea pityocampa* (Aribi and Soltani, 1992) and *Pieris brassicae* (Lafont et al., 1974).

Change in ecdysteroid amounts of *C. pipiens* showed a single peak that occurred at day three. The number of ecdysteroid peaks detected varied depending on the stages and the insect species. In some non-dipteran species such as *Tenebrio molitor*, *P. brassicae* or

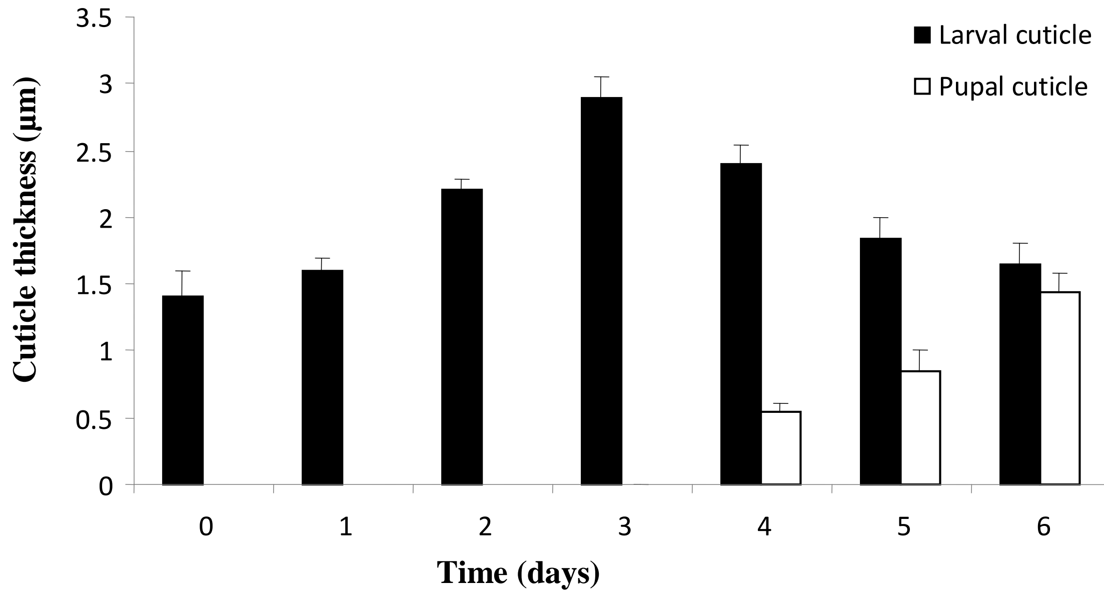

Figure 3. Changes in larval and adult cuticle thickness during the fourth instar larval development of *C. pipiens* (mean ± SD based on five repeats per time).

Galleria mellonella, the last larval stage presents two peak of secretion (Dean et al., 1980), while a single peak was observed in larvae of *Zophobas atratus* (Aribi et al., 1997), *T. pityocampa* (Aribi and Soltani, 1992) or in *Bombyx mori* and *S. gregaria* (Dean et al., 1980). During the pupal-adult development, generally, a single peak was secreted (Delbecque et al., 1978; Soltani et al., 1989a, 2002), and a second peak produced by ovaries was reported in some species such as *Cydia pomonella* (Soltani, 1986; Soltani et al., 1989b).

There is a correlation between the phases of an ecdysteroid peak and the various physiological and morphological events in the moulting cycle (Nijhout, 1994). In the present study, change in the cuticle thickness during the last larval stage of *C. pipiens* showed that the larval cuticle, that is, the old cuticle increased to reach a maximum at day three, which coincided with the moment of the ecdysteroid titer peak and the start of apolysis. Further, the thickness decreased as the old cuticle started to be re-absorbed, reaching a low value at day six, which is the day of pupal ecdysis. The deposition of the pre-ecdysial cuticle, that is, the new pupal cuticle, starts immediately after apolysis. Thus, the ecdysteroid peak recorded during the 4th-instar larval development coincides with the apolysis and could be related with the induction of a new cuticle secretion, confirming previous reports made on other species (Delbecque et al., 1978; Soltani, 1986; Soltani et al., 1989a; Aribi and Soltani, 1992; Bendjedou et al., 1998; Rehimi and Soltani, 1999). Thus, according to Nijhout (1994), the moulting event of apolysis concurs with a rise in ecdysteroids, and the new cuticle deposition starts during the time when the maximum of the peak is reached. According to Lafont et al. (2005), free and conjugated forms of ecdysteroids are confirmed in several insects. Whole body extracts of *C. pipiens* may contain considerable amounts of conjugated ecdysteroids. Therefore, the present study must be completed by other experiments such as the determination of different ecdysteroid forms in larval body extracts.

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