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Full Length Research Paper

Enzyme immunoassay measurements of the molting hormone in different post-embryonic stages of two mosquito species, *Culex pipiens* and *Culiseta longiareolata*

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Due to their large geographical distribution, abundance and harmfulness, *Culex pipiens* L. and *Culiseta longiareolata* Macquart are the two most remarkable mosquito species in Algeria. The nature and the level of ecdysteroids from whole body extracts of different post-embryonic stages of the two mosquito species was made 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 with a strong affinity for ecdysone (E). EIA measurements confirmed the presence of two main hormones in the two mosquito species: E and 20E. Moreover, there is a predominance of 20E.

Key words: Mosquitoes, *Culex pipiens*, *Culiseta longiareolata*, hormone, enzyme-immunoassay (EIA).

INTRODUCTION

Mosquitoes are vectors of several pathogens such as protozoa, viruses and nematodes that are transmitted to humans and pets (Nuttall, 1997). Due to their large geographical distribution, abundance and harmfulness, *Culex pipiens* L. and *Culiseta longiareolata* Macquart are the two most remarkable mosquito species in Algeria (Rehimi and Soltani, 1999; Tine-Djebbar and Soltani, 2008). The phenomena of molting and metamorphosis of insects are controlled by different hormones that are essentially the moulting hormone (ecdysteroids) secreted by the prothoracic glands, and juvenile hormone (JH), produced by the corpora allata (Dhadialla et al., 1998). The moulting hormone plays a role during reproduction, affecting among others, the development of oocytes. Thus, it is necessary for the initial growth of the oocyte and follicle differentiation of immature stage vitelline (Laverdure, 1972). Other functions have been attributed to ovarian ecdysteroids in different species of insects such as control of meiotic reinitiation in oocytes (Lanot et al., 1985), ovulation induction of sclerotization of the egg, stimulating the formation of the chorion, the contribution of a source of ecdysteroids necessary for embryonic moults to the embryo (Lagueux et al., 1977) and inhibition of production of juvenile hormone (Lanot et al., 1985). The regulation of various physiological parameters of reproduction in insects is dependent on the endocrine system. Ovarian growth, ovulation and oviposition are under the control of three major groups of hormones: juvenile hormones, the ecdysteroids and neurohormones (Adams and Fillipi, 1988; Hoffmann et al., 1999). The molting hormones are among the most important regulators of insect development. As a result of the primary role of ecdysteroids in the development and reproduction of insects, these hormones are a new target for various growth regulators (Dinan, 1989). Data on ecdysteoids constitute an experimental basis to investigate new selective insecticides such as the ecdysteroid agonists against mosquitoes. Therefore, the present study aimed to determine the nature and the levels of ecdysteroids by an enzyme immunoassay (EIA) in immature stages and adult males and females of *C. pipiens* and *C. longiareolata*, two mosquito species showing medical and veterinary importance, respectively.

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Table 1. Mean body weight (mg) of individuals from different newly ecdysed instars of *C. pipiens* and *C. longiareolata* (m ± SD, established on 5 replicates each containing 25 individuals per instar).

Instar	*Cx pipiens*	*Cs longiareolata*
4th-instar larvae	3.77 ± 0.87[a]	11.59 ± 0.28[b]
Pupae	2.90 ± 0.62[a]	7.95 ± 1.70[b]
Male adult	1.77 ± 0.05[a]	4.09 ± 0.13[b]
Female adult	2.92 ± 0.12[a]	6.23 ± 0.73[b]

For each instar, mean values followed by different letters are significantly different ($p<0.05$).

Moreover, the results determined the appropriate antibody for use in further measurements of this hormone in these two mosquito species.

MATERIALS AND METHODS

Insects

C. pipiens and *C. longiareolata* (Diptera, Culicidae) were obtained from a stock colony and kept as previously described by Rehimi and Soltani (1999). Pyrex storage jars (80 by 100 mm) containing 150 ml of tap water were maintained at temperature of 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. The mean body weight of individuals from different newly molted instars of *C. pipiens* and *C. longiareolata* was established on 5 repeats, each corresponding to a pool containing 25 individuals per instar. To reduce the variability in each bioassay, samples of each instar were collected at the same age (just after the exuviations and <4 h old).

Ecdysteroid extraction

The quantification of ecdysteroids was made in fourth instar larvae, pupae, of male and female adults from two mosquito species *C. pipiens* and *C. longiareolata*. Individuals were collected and pooled samples were subjected to extraction of free ecdysteroids. After individual extraction with methanol by sonication, the samples were centrifuged at 5,000 g for 10 min, and the supernatants were taken and evaporated.

Enzyme immunoassay for ecdysteroids

Each whole body extract was suitably resuspended in phosphate buffer (0.1 M, pH 7.4) and analyzed in duplicate by an EIA as previously described (Soltani et al., 2002) using a conjugate of an antibody against 20E coupled to peroxidase as an enzymatic tracer and tetramethyl benzidine as a color reagent.

The nature of ecdysteroids was determined by the use of two specific antibodies kindly supplied by Dr. J. P. Delbecque, University of Bordeaux, France, a rat monoclonal EC 19 antibody showing high affinity for 20E was used for measurements of 20E only, whereas a rabbit polyclonal B antibody more sensitive to E (6 times more sensitive to E than to 20E) (De Reggi et al., 1992) was used for analysis of both E and 20E. The quantification of hormone was determined using serial dilution of E or 20H (Sigma, France) as standards. Data are expressed as pg ecdysteroid equivalents per individual or per mg whole body.

The relative importance (%) of different hormones is presented after the correction of the antibody immuno-reactivity. EIA measurements were done on five repeats per instar, each corresponding to a pool containing 25 individuals and each was analysed in duplicate.

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, PA State College, USA). The number of individuals tested in each bioassay is given in the results.

RESULTS

The mean body weight of individuals from different newly molted instars of *C. pipiens* and *C. longiareolata* are presented in Table 1. The body weight of individuals shows a slight decrease during the post-embryonic development of the two studied mosquito species. Moreover, the comparison of the mean body weight of each stage revealed a significant ($p < 0.0012$) difference between the two species. The highest values were recorded in *C. longiareolata*. The body weight of adults was significantly high in females than in males in *C. pipiens* ($p< 0.0032$) and in *C. longiareolata* ($p< 0.0008$).

The nature and amount of free ecdysteroids in newly molted stages of *C. pipiens* and *C. longiareolata* was tested by an EIA using two antibodies. The quantification was made by comparison with reference curves each, established with serial concentration of E and 20E as standards. Tables 2 and 3 show the contents and the amounts of ecdysteroids, respectively. Data show the presence of two main hormones: E and 20E. Generally, the hormonal contents decreased slightly during the larval-pupal-adult development in the two mosquito species (Tables 2). 20E, the active form, was the major hormone in all body extracts from the two studied species. In addition, significantly ($p< 0.0249$) high contents of these two ecdysteroid hormones were detected in *C. longiareolata* in comparison with *C. pipiens* (Tables 2 and 3). The relative importance (%) of different hormones is presented after the correction of the antibody immunoreactivity (Table 4). The immuno-reactivity was corrected since the rabbit polyclonal B

Table 2. Contents of ecdysone and 20-hydroxyecdysone (pg/individual) in newly molted fourth-instar larvae, pupae and adults of *C. pipiens* and *C. longiareolata.* Data are expressed as means ± SD, established on 5 replicates each containing 25 individuals per instar).

Instar	20-hydroxyecdysone (pg equi. 20E /ind.)		Ecdysone (pg equi. E /ind)	
	C. pipiens	***C. longiareolata***	***C. pipiens***	***C. longiareolata***
4th-instar larvae	0.57 ± 0.13^{a}	0.63 ± 0.06^{b}	1.58 ± 0.69^{a}	2.61 ± 0.13^{b}
Pupae	0.15 ± 0.04^{a}	0.43 ± 0.06^{b}	0.51 ± 0.14^{a}	1.39 ± 1.15^{b}
Male adult	0.01 ± 0.006^{a}	0.11 ± 0.011^{b}	0.07 ± 0.06^{a}	0.13 ± 0.05^{a}
Female adult	0.03 ± 0.01^{a}	0.45 ± 0.43^{b}	0.08 ± 0.00^{a}	0.76 ± 0.14^{b}

For each hormone and each instar, mean values followed by different letters are significantly different ($p<0.05$).

Table 3. Amounts of ecdysone and 20-hydroxyecdysone (pg/mg body weight) in larvae, pupae and adult males and females of *C. pipiens* and *C. longiareolata* using a monoclonal and polyclonal antibody (m ± SD, established on 5 replicates each containing 25 individuals per instar).

Instar	20-hydroxyecdysone (pg equi. 20E /mg)		Ecdysone (pg equi. E /mg)	
	C. pipiens	***C. longiareolata***	***C. pipiens***	***C. longiareolata***
4th-instar larvae	0.15 ± 0.02a	0.06 ± 0.05b	0.50 ± 0.14a	0.21 ± 0.02b
Pupae	0.05 ± 0.01a	0.05 ± 0.01b	0.18 ± 0.03a	0.17 ± 0.12b
Adult male	0.01 ± 0.004a	0.03 ± 0.01b	0.03± 0.01a	0.08 ± 0.01b
Adult female	0.02 ± 0.006a	0.07 ± 0.01b	0.04 ± 0.01a	0.12 ± 0.04 b

For each hormone and each instar, mean values followed by different letters are significantly different ($p<0.05$).

Table 4. Relative importance of ecdysteroids (%) in larvae, pupae and adult males and females of *C. pipiens* and *C. longiareolata* after the correction of the antibody immuno-reactivity (m ± SD, established on 5 repeats each containing 25 individuals per instar).

Instar	Ecdysone (%)		20-hydroxyecdysone (%)	
	C. pipiens	***C. longiareolata***	***C. pipiens***	***C. longiareolata***
4th-instar larvae	27.90 ± 5.20^{a}	20.70 ± 1.40^{b}	72.10 ± 3.10^{a}	79.30 ± 3.40^{b}
Pupae	21.73 ± 0.38^{a}	22.72 ± 1.57^{a}	78.26 ± 0.26^{a}	77.27 ± 1.57^{a}
Male adult	20.00 ± 2.10^{a}	27.27 ± 3.33^{b}	80.00 ± 1.61^{a}	72.72 ± 3.33^{b}
Female adult	25.00 ± 6.75^{a}	36.84 ± 2.38^{b}	75.00 ± 5.99^{a}	63.15 ± 2.38^{b}

For each hormone and each instar, mean values followed by different letters are significantly different ($p<0.05$).

antibody was 6 times more sensitive to E than to 20E. The percentage of 20E varied as function of the stage, from 72 to 80% in *C. pipiens* versus 63 to 79% in *C. longiareolata*. In adult stage, the percentage of ecdysone was high in females as compared to males, and also in *C. longiareolata* adults as compared to *C. pipiens* adults (Table 4).

DISCUSSION

The Culicidae of Mediterranean Africa was the subject of a recent inventory (Bruhnes et al., 2000). In Algeria, *C. pipiens* presents a medical and environmental interest. The ecdysteroids, polyhydroxylated steroids play an important role in controlling several processes such as growth, metamorphosis and reproduction (Rees, 1995). Their qualitative and quantitative analysis is essential (Pascual et al., 1995).

As reviewed by Hagedorn (1985) and Telfer (2009), in the adult female, ecdysteroids are synthesized by the follicle cells in the ovaries and play a major role in ovarian development, vitellogenesis and oocyte/egg maturation. The prothoracic glands are the site of synthesis of ecdysteroids after their stimulation by a neurohormone secreted by the cephalic neurosecretory cells, the hormone prothoracicotrope. The prothoracic glands develop and become functional during the last stage of

embryogenesis. They are active in the larval stage but degenerate in many insect species after the last molt during the larval or adult stage (Romer, 1971; Glitho et al., 1979; Aribi et al., 1997) and the existence of other hormonal sources has been demonstrated (Gersch, 1978; Soltani et al., 1989; Delbecque et al., 1990; Jenkins et al., 1992) as the ovaries (Goltzene et al., 1978; Hoffman et al., 1992; Hofsteenge et al., 1994, Soltani et al., 1997), oenocytes and sternites of insects (Delbecque et al., 1990; Asahina et al., 1994), and the epidermis or epithelial cell lines or wings (Delbecque et al., 1990). This information on the moulting hormone constitute an experimental basis to investigate new selective insecticides such as the ecdysteroid agonists against mosquitoes.

EIA measurements of free ecdysteroids in whole body extracts of two mosquito species, *C. pipiens* and *C. longiareolata* revealed the presence of two major hormones: E and 20E. 20E, the active form, was the major hormone in all body extracts. 20E is more abundant in larvae and pupae of *C. pipiens* as compared to *C. longiareolata*. The predominance of this hormone has also been reported in some species such as *Pieris brassicae* (Lafont et al., 1974), *Manduca sexta* (Bollenbacher et al., 1975), *Schistocerca gregaria* (Morgan and Poole, 1976), *Locusta migratoria* (Hirn et al., 1979), *Calpodes ethlius* (Dean et al., 1980), *Cydia pomonella* (Soltani, 1986) or *Thaumetopoea pityocampa* (Aribi and Soltani, 1992). In mosquitoes, Aedes, the ecdysteroids are secreted by the ovaries and are able to control the synthesis of yolk (Fallon et al., 1974; Huybrects and De Loof, 1997; Postlethwait et al., 1980). The 20-hydroxyecdysone ensures separation of the second follicle germanium (Beckmeyer and Lea, 1980). The concentration of ecdysteroids undergoes intense changes during insect development (Shaaya and Karlson, 1965). Conclusively, the overall results showed that 20E is the major ecdysteroid hormone in body extracts of *C. pipiens* and *C. longireolata*. Thus, the monoclonal antibody is more appropriate for use in further EIA measurements of ecdysteroids in these two mosquito species.

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