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EFFECTS OF PRECOCENE AND AZADIRACHTIN IN *RHODNIUS PROLIXUS*: SOME DATA ON DEVELOPMENT AND REPRODUCTION

ELOI S. GARCIA, DENISE FEDER, JOSÉ EUGÉNIO P.L. GOMES & PATRICIA DE AZAMBUJA*

Instituto Oswaldo Cruz, Departamento de Bioquímica e Biologia Molecular, Caixa Postal 926, 20001 Rio de Janeiro RJ, Brasil *Departamento de Biologia Geral, Universidade Federal Fluminense, Caixa Postal 183, 24001 Niterói, RJ, Brasil

The results presented in this paper clearly indicate that precocene and azadirachtin are effective inhibitors of moulting and reproduction in the hemipteran Rhodnius prolixus. *The time of application is important and only applications of these substances early in the intermoulting period cause their effects in nymphs. The inhibition of moulting is fully reversed by ecdysone therapy. Precocene and azadirachtin also affected drastically the oogenesis and eggs deposition in this insect. Precocene-induced sterilization is reversed by application of juvenile hormone III. However, this hormone is unable to reverse the effect of azadirachtin on reproduction. Ecdysteroid titers in nymphs and adult females are decreased by these treatments.* In vitro *analysis suggest that precocene and azadirachtin may act directly on the prothoracic glands and ovaries producing ecdysteroids. Based on these and other findings the possible mode of action of these compounds on the development and reproduction of* Rhodnius prolixus *is discussed.*

The endocrine system that controls development and reproduction in insects has many unique features that could provide targets for compounds such as precocene and azadirachtin which apparently disrupt the biosynthesis and/or release of hormones in insects. These substances produce juvenile hormone and/or ecdysone deficiency symptons in the animal (for review see Bowers, 1982a, b; Rembold, 1984). In the search for models to study the effects of the above mentioned substances on insects, we have used the hemipteran hematophagous *Rhodnius prolixus* as a suitable animal model and a very powerful tool for this purpose. This insect is particularly adequate for research on compounds which specifically or not target onto the insect's own endocrine system, because of the following reasons: (i) information already available on the general basic physiology and specially on endocrine system; (ii) synchronization of development and reproduction by a blood meal; (iii) high sensitivity to hormones of insect; (iv) easily reared and handled on a large-scale in the laboratory (for review see Buxton, 1930; Wigglesworth, 1969, 1970, 1972, 1973; Langley & Pimley, 1978; Gardiner & Maddrell, 1972; Slama et al., 1974; Novak, 1975; Garcia et al., 1975; 1984a, b, c, 1986, 1987; Tarrant et al., 1982; Garcia & Azambuja, 1987).

This paper describes the alterations in development and reproduction induced by precocene (classically known as antijuvenile hor-) mone) and azadirachtin (a potent insect growth regulator) in *Rhodnius prolixus* as well as the usefulness of this insect as a simple model to gather infomation on the biological effects of these compounds on insects.

1. *Research on moulting*

An elegant and efficient proof of the effect of coumpounds on immature insects is the alteration of the moulting cycle since almost all of the observed effects so far appear to be related to a deficiency of circulating hormone involved in ecdysis. Despite the abundance of research (for review see Bowers, 1976, 1982a, b; Bowers et al., 1976; Rembold et al., 1983; Staal, 1986) several aspects of the effects of such substances are far from being understood. This gap in knowledge is complicated as many of these compounds appear to have multiple actions on the insect. We discuss here how these compounds interfere with the moulting cycle, the counteraction of this activity by simultaneous application of ecdysone, and the effects on the ecdysteroid titers in the hemolymph of *in vivo* treated nymphs.

1.1. Sensitive period during moulting cycle — It seems that precocene and azadirachtin

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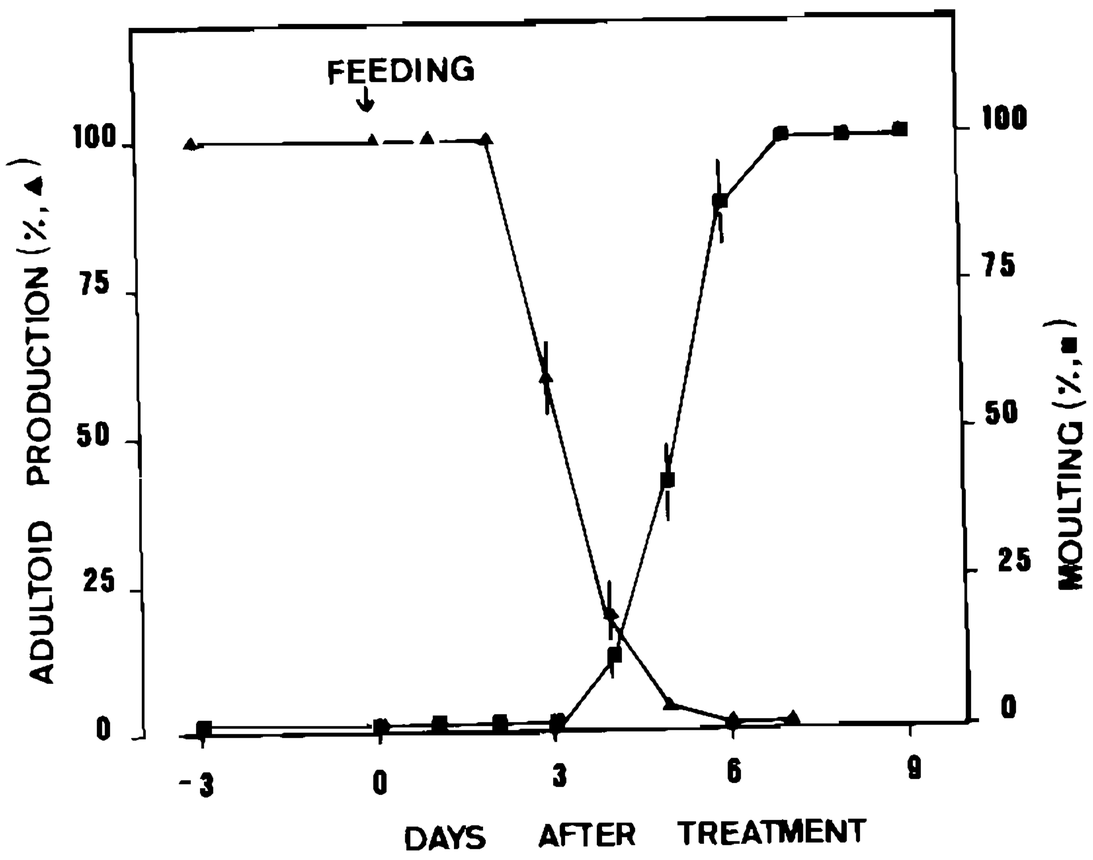

Fig. 1: Effects of ethoxyprecocene II and azadirachtin A applied at various time before and after a blood meal (day zero), on the production of adultoid and inhibition of moulting in 4th-instar nymphs of *Rhodnius prolixus*, respectively. Ethoxyprecocene II (60 µg/nymph, ▲) was topically applied (5 µl). Azadirachtin A (15 ng/nymph, ■) was given by injection (1 µl). Each point represents results from groups of at least 15 insects.

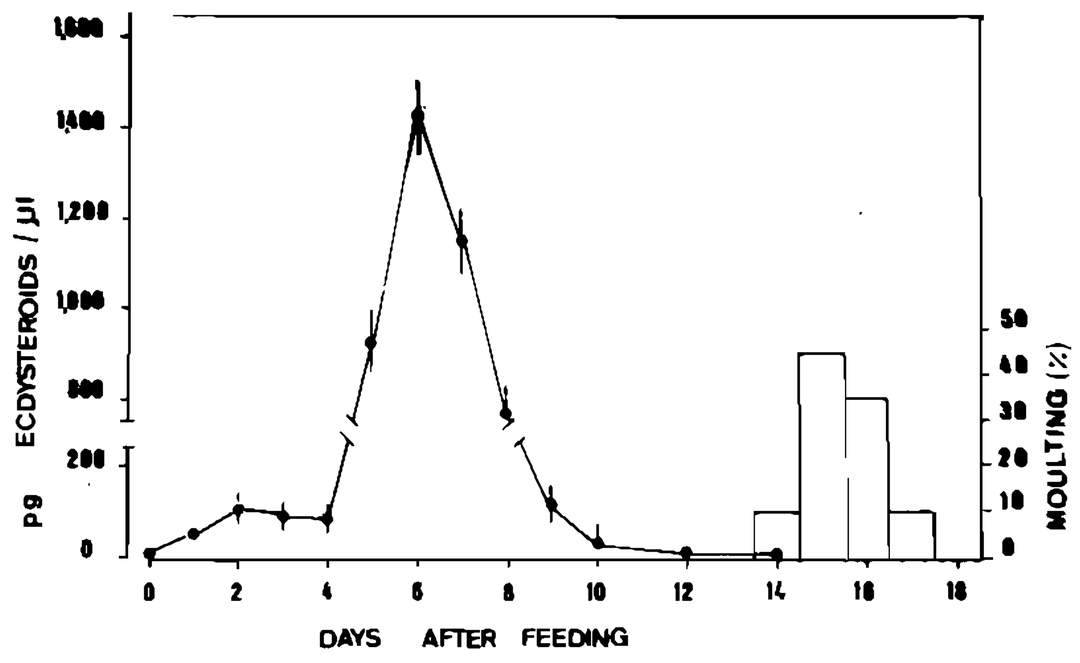

Fig. 2: Hemolymph ecdysteroid concentrations of 4th-instar nymphs of *Rhodnius prolixus* at various times after a blood meal (●). Each point is the meam of 6-8 determinations ± S.D. (one per nymph). Histograms on right illustrate the period of ecdysis for 5th-instar nymphs of 40 insects synchronized by a blood meal on day zero. Radioimmunoassay (RIA) for ecdysteroids was carried out according to the methods of Chang & O'Connor (1979) and Sourmoff et al. (1981). Ecdysteroid antiserum was a gift of Dr. J. D. O'Connor (UCLA, USA).

exert their physiological effects by arresting juvenile hormone and ecdysteroid biosynthesis. If this is true, timing is essential and only applications of these compounds early in the intermoulting period will cause their physiological effects. To establish the limits of the sensitive period the responses of 4th-instar nymphs of *Rhodnius prolixus* to ethoxyprecocene II (the classical production of adultoids) and to azadirachtin A (inhibition of moulting) were determined (Fig. 1). The production of adultoids was maximal when precocene was applied before feeding until day 3 after a bloodmeal. On the 4th day this treatment had no effect on the production of precocious metamorphosis. Fig. 1 also shows that azadirachtin induced a drastic inhibition when applied from before feeding to day 3 after feeding, while the treatment between day 7 and 9 had no effect on moulting. The group with inoculation of azadirachtin on day 4 to 6 had the ecdysis only partially inhibited (for details see Azambuja & Garcia, 1987; Garcia et al., 1986). Thus, the sensitive period ends day 4 for these compounds, i. e., at the time when ecdysteroid peak in the hemolymph begins (compare Fig. 1 with Fig. 2). The exact limits of the sensitive period, for both compounds, however, cannot be determined with accuracy even in timed insects, synchronized by a bloodmeal on day zero, due to a developmental asynchrony presented by them at least regarding ecdysis. The histogram included in Fig. 2 shows that a population of 4th-instar nymphs may differ by as much as 4 day for moulting. On the basis of the present findings, the sensitive period is then of 4 days after feeding, at least for the developmental pathway dictated by juvenile hormone and ecdysone expression.

1.2. Moulting arresting and ecdysial stasis – For all practical purposes, assays of precocene and azadirachtin on immature insects are the easiest and most relevant. Nymphs of *Rhodnius prolixus* may respond with either precocious metamorphosis and partial inhibition of moulting to precocene (Tarrant & Cupp, 1978, Azambuja et al., 1981a, b; Garcia et al., 1984a; Garcia & Azambuja, 1987) or entirely ecdysial stasis to azadirachtin (Garcia & Rembold, 1984; Garcia et al., 1984b, 1986). The arresting and ecdysis inhibition induced by these compounds could be reversed by therapy with ecdysone (Azambuja et al., 1981a, b; Garcia et al., 1984a, b). It is reasonable to speculate that these compounds would act directly and/or indirectly on the ecdysone production by prothoracic glands.

With this idea in mind our attention was drawn to the ecdysteroid levels in the hemolymph of normal and nymphs treated with precocene and azadirachtin, as a test of the above hypothesis. Unfed 4th-instar nymphs have a very low ecdysteroid titer. Soon after feeding the titers rise and stabilize until day 4. The hormone titer begins to increase on day 5

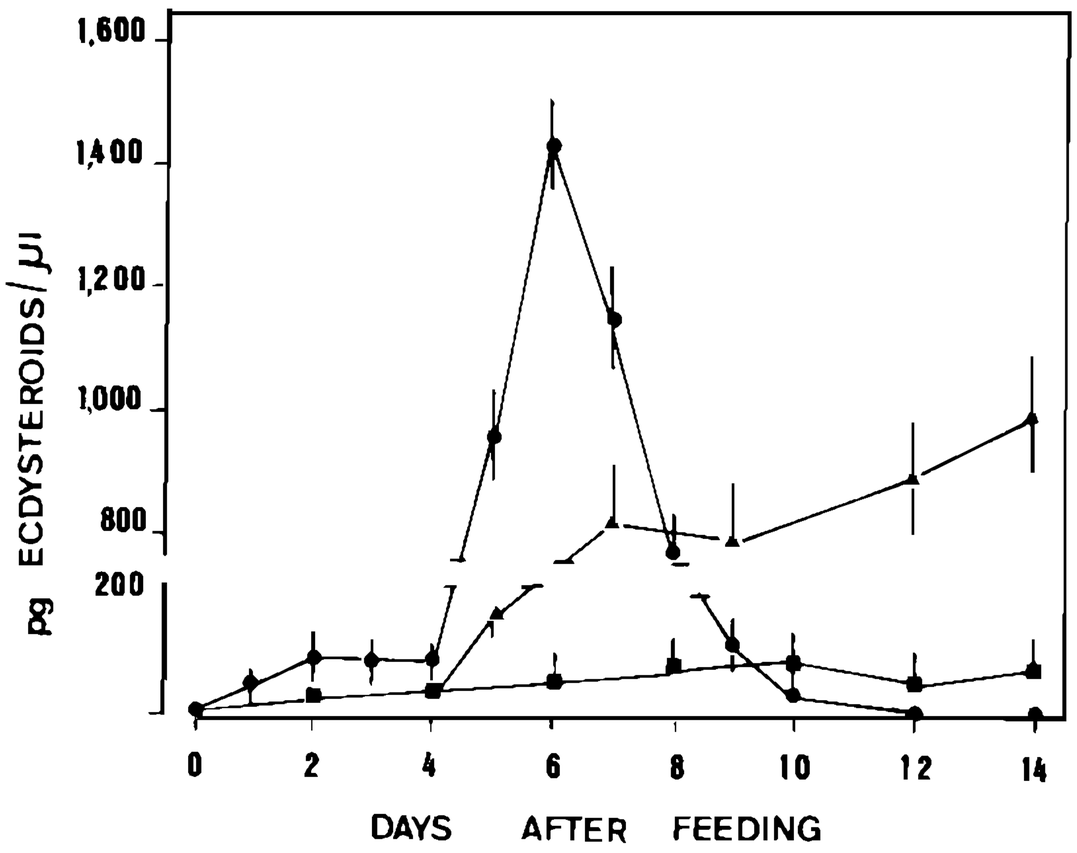

Fig. 3: Hemolymph ecdysteroid titers in 4th-instar nymphs of *Rhodnius prolixus* controls (●), treated with ethoxyprecocene II (60 µg/nymph, ▲) or azadirachtin A (15 µg/nymph, ■). Each point from controls is the mean of at least 6 individual determinations. The other points represent pooled hemolymph from 5 insects (4 determinations). RIA as described in Fig. 2.

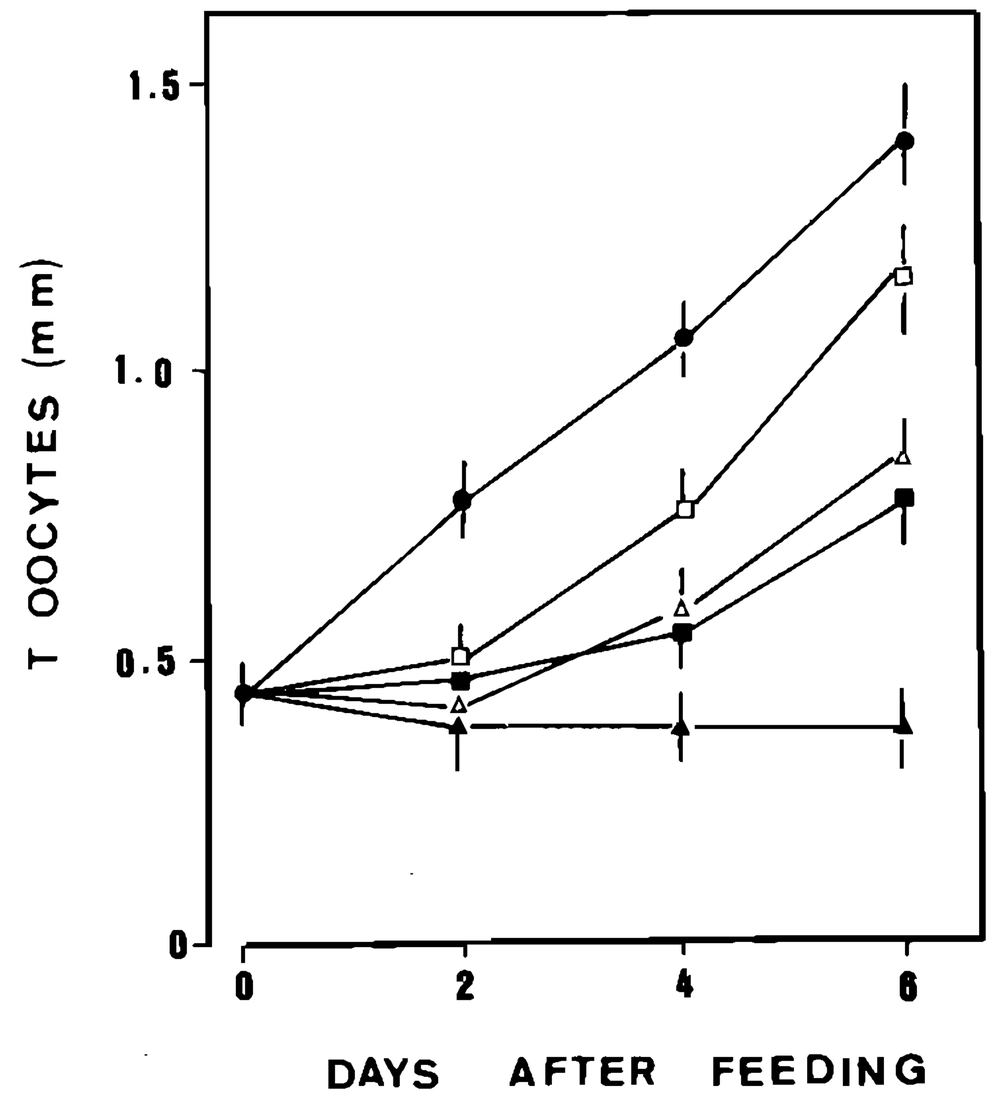

Fig. 4: Effects of ethoxyprecocene II (1.0 µg, △, and 10.0 µg/ml of blood meal, ▲) and azadirachtin A (0.5 µg/ml of blood meal, ■) on the length of terminal oocytes, 0,6 days following feeding. Each value represents the mean ± S.E. of the T oocytes present in both ovaries of the 5 females.

reaching the highest levels during the intermoulting period on day 6, and then the titer falls rapidly to background levels ecdysis (Figs. 2 and 3). Analysis of the ecdysteroid levels in nymphs treated with precocene did not reveal a well defined ecdysteroid peak and the hormonal titers were very small when compared with the controls (Fig. 3). The ecdysteroid titers were drastically reduced and remained at very low levels during the entire experimental period of observation in the nymphs receiving azadirachtin (Fig. 3). As ecdysone play a very important role in inducing ecdysis our results also support the hypothesis of the precocene and azadirachtin act directly and/or indirectly on the glands which produce multing hormone.

2. *Research on reproduction*

Since the regulation of reproduction in insects by hormones is well known (see Wigglesworth, 1970, 1972; Novak, 1975; Slama et al., 1974) another evaluation for the action of compounds which interfere with the endocrine system of insects is the effect on oogenesis of adult females. The effects of precocene and azadirachtin on ovary maturation may also be explored using adult female of *Rhodnius prolixus* as a model.

2.1. Oocyte growth and egg production – As a preliminary study the oocyte development (Terminal (T) oocyte) was measured as an effective determination of vitellogenesis. The ingestion of precocene and azadirachtin prevented yolk deposition and the T oocytes did not show any significant increase, i. e, practically eggs were not produced in the higher doses. The insects treated with low doses of both compounds had only a small increase of the gonads (Fig. 4). Precocene and azadirachtin drastically inhibited the production of eggs (Table I). However, while the inhibition of egg deposition in precocene-treated insects was counteracted by therapy with juvenile hormone III, the same did not occurr for the treatment with azadirachtin (Table I). It seems that azadirachtin affects *Rhodnius* oogenesis independently of juvenile hormone production.

2.2. Ecdysteroid titers – In many insects juvenile hormone and ecdysone play a role in vitellogenesis (Hagedorn, 1983). In *Rhodnius prolixus* virtually nothing is known about possible control of vitellogenin synthesis by ecdysteroids. However, it is well known that juvenile

TABLE I

Average rate of egg production during the first cycle of oviposition in females that received different doses of ethoxyprecocene II and azadirachtin A (*).

Compounds	µg/ml of blood	JH III (**) µg/female	eggs/female/day
None (control)	–	–	1.20 ± 0.25
Ethoxyprecocene II	1.0	–	0.51 ± 0.12
	10.0	–	0.31 ± 0.05
Azadirachin A	0.5	–	0.78 ± 0.17
	5.0	–	0.55 ± 0.14
Ethoxyprecocene II	10.0	1.0	1.10 ± 0.32
Azadirachtin A	5.0	1.0	0.62 ± 0.25

(*) Each number is the mean ± S.E. of at least 10 females. (**) Juvenile hormone III was topically applied.

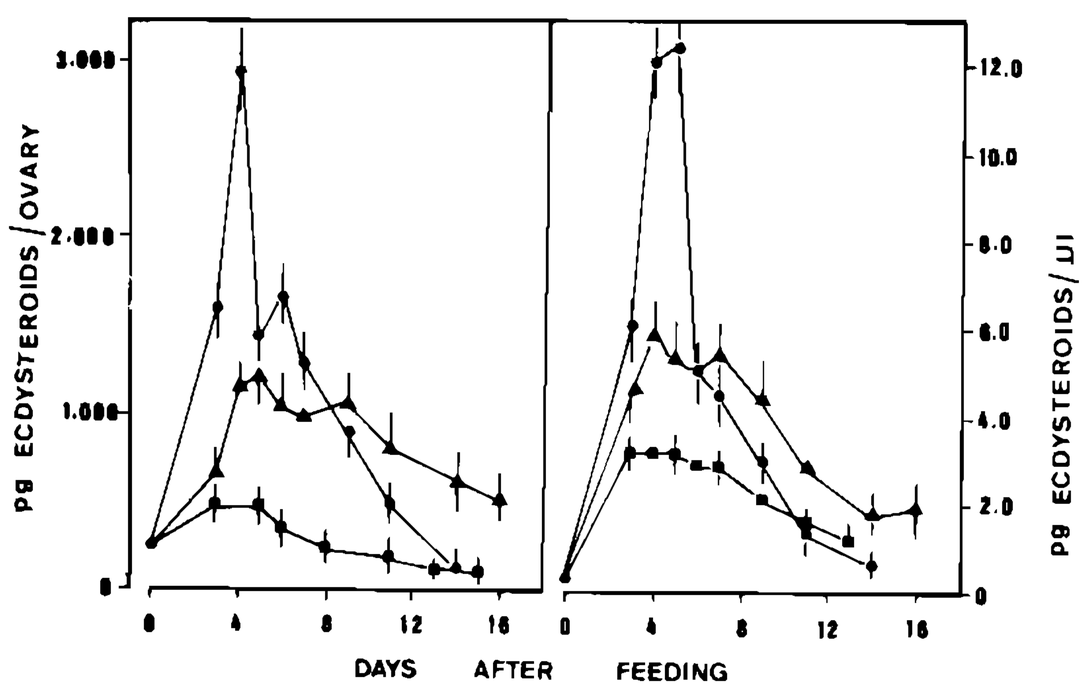

Fig. 5: Effect of azadirachtin A (5 µg/ml of blood meal) and ethoxyprecocene II (10 µg/ml of blood meal) on the total ecdysteroid of ovary (A) and hemolymph (B). The results are based on ovary measurements of individual females (4 determinations) and pooled hemolymph of 3 insects (4 determinations). ●, controls; ■, azadirachtin A; ▲, ethoxyprecocene II. Ria as described in Fig. 2.

hormone is needed (Wigglesworth, 1972; Davey, 1980). The presence of ecdysteroids in adult females of insects is well recognized (Hoffman et al., 1980), but their functions still need further study. Mature ovaries of insect which produce ecdysteroids are, for example, responsible for reduced activity of the *corpus allatum* (Stay et al., 1980). This hypothesis is supported by the finding that administration of ecdysteroids to insect reduced the production of juvenile hormone (Tobe & Stay, 1981) and egg production (Garcia et al., 1979). Because the ecdysteroids are also produced by the mature ovaries of *Rhodnius prolixus* (Ruegg et al., 1981) and are involved at least as a modulator of electrical activity in neurosecretory cells (Ruegg et al., 1982) and possibly in the ovulation (Orchard et al., 1983), the question arises of a possible relationship between ecdysteroids in adult females and the effects of precocene and azadirachtin on reproduction. The titers remain unchanged for the first 2 days after feeding and then rise sharply to a peak on day 4 in the ovary and on day 5 in hemolymph, decreasing rapidly later (Fig. 5). It is interesting to observe that the peak value of ecdysteroids in the ovary is aproximately 3,000 pg/ovary, while in the hemolymph is 18 pg/µl. Assuming that the volume of hemolymph in adult *Rhodnius* is 30 - 40 µl, only small amounts of ecdysteroids are being released from the ovary into the hemolymph. The groups treated with precocene and azadirachtin failed to show the normal increase in ecdysteroid titers in ovary and hemolymph (Fig. 5). However, while in precocene-treated insects the hormonal titers had a small increase in both tissues, the treatment with azadirachtin presented only background titers in ovary and hemolymph. These data suggest that, in some way, these compounds are inhibiting, directly and/or indirectly, the main site of the production of ecdysteroids found in adult females.

TABLE II

Spontaneous synthesis of ecdysteroids by prothoracic glands and ovaries of *Rhodnius prolixus*. Prothoracic glands, derived from either control or treated fourth-instar nymphs, were removed day 6 after feeding. Ovaries were taken day 4. They were incubated in TC 199 medium containing or not ethoxyprecocene II or azadirachtin A at 28°C

Compounds	Treatments in vivo µg/ml blood	Treatments in vitro µg/ml medium	ecdysteroid production pg/gland/h
Prothoracic glands	–	–	120 ± 15 (*)
	Ethoxyprecocene II 20.0	–	30 ± 7
	Ethoxyprecocene II –	10.0	45 ± 9
	Azadirachtin A 1.0	–	25 ± 5
	Azadirachtin A –	1.0	50 ± 9
Ovaries	–	–	820 ± 75
	Ethoxyprecocene II 5.0	–	350 ± 40
	Ethoxyprecocene II –	5.0	410 ± 65
	Azadirachtin A 1.0	–	110 ± 12
	Azadirachtin A –	1.0	150 ± 17

(*) Each point represents the mean ± S.E. of 5 separate determinations.

3. *In vitro research*

In vitro analysis of ecdysteroid biosynthesis in intact prothoracic glands and ovaries in culture medium can give insights into the mechanism of action of precocene and azadirachtin in insects. These glands spontaneously introduced ecdysteroids into the medium during *in vitro* cultures (Gruetzmacher et al., 1984a, b; Rubesnstein et al., 1982; Garcia et al., 1987; Garcia & Azambuja, 1987). *In vitro* synthesis of ecdysteroids in control adult females is illustrated in Table II. Ovary and prothoracic glands removed from insects treated *in vivo* with precocene and azadirachtin presented a drastic inhibition of ecdysteroid synthesis. The incubation of either normal ovary or prothoracic glands in a medium containing ethoxyprecocene II and azadirachtin A significantly reduced ecdysteroid production (Table II). The relevance of the *in vitro* inhibition of the prothoracic glands and ovaries compared to the overall effects *in vivo* is often difficult to establish since these compounds may act simultaneously on other target systems. Although ecdysteroids are important in inducing moulting in nymphs and there is little or no evidence that ecdysteroid is relevant for *Rhodnius* oogenesis our *in vitro* studies indicate that precothese hormones. It is unknown, however if these *in vitro* effects are due to high concentrations of the compounds in the culture media.

Concluding remarks

In this paper, whenever possible, the effects of precocene and azadirachtin were studied in terms of the usefulness of *Rhodnius prolixus* to elucidate how these compounds disrupt development and reproduction in insects. It should be kept in mind that, although precocene and azadirachtin are known for more than 10 years, several aspects of their actions on insect are unclear and raise a great many questions of a fundamental biological and biochemical nature that invite future investigations. In this study it was possible, for example, to compare the activities of such compounds on the whole organism by measuring the sensitive period, arresting and inhibiting moulting, producing adultoids as well as sterilizing adult females. Emphasis was given to the ecdysial stasis caused by precocene and azadirachtin and the reversal of this effect by ecdysone therapy (Azambuja et al., 1981a, b; Garcia et al., 1984a; Garcia & Rembold, 1984; Garcia & Azambuja, 1987). The impact of these compounds on the production of eggs was also shown. Drastic sterilization could be observed by treatment with precocene and azadirachtin. The effect of the former compound is reversed by simultaneously treatment with juvenile hormone III. Special attention was placed in the production of ecdysteroids by prothoracic glands in nymphs and by ovaries in adult females. The increase of ecdysteroid titers in hemolymph was either partially (in precocene-treated insects) or entirely (in azadirachtin-treated animals) inhibited. Similar data were observed when prothoracic glands and ovaries were incubated in a medium containing these compounds suggesting that the direct effect of precocene and azadirachtin on the glands produces ecdysteroids.

The effects of precocene inducing precocious metamorphosis and sterilization are very well known (Bowers, 1976; Bowers et al., 1976). However, the inhibition of moulting by this compound is poorly understood. It is believed that precocene acts directly on prothoracic glands limiting or abolishing ecdysteroid synthesis (Garcia & Azambuja, 1987; Garcia et al., 1987; present paper). Similar findings are described by azadirachtin action on moulting (present paper). The indirect effect of precocene (Bowers, 1976; Unnithan et al., 1978; Azambuja et al., 1981b) and azadirachtin (Rembold et al., 1983; Sieber & Rembold, 1983; Garcia et al., 1986) on the neurosecretory cells from the brain is inferred for explaining the effects of these compounds on moulting and reproduction of insects.

Notwithstanding these other regulatory considerations, our findings give emphasis to the direct effect of precocene and azadirachtin on the prothoracic glands and ovaries affecting the ecdysteroid production and the hormonal interaction mediated by this hormone. Whether or not these compounds also act by interfering with ecdysone metabolism and/or the general metabolic pathway are problems to be solved by future research.

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