Azadirachtin-Induced Sterilization in Mature Females of *Rhodnius prolixus*

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The effects of azadirachtin A, given orally, on the reproduction of mature females of *Rhodnius prolixus* were studied. Azadirachtin A caused a reduction in oocyte growth and consequently in the egg production in a dose-dependent manner. Eggs layed had no alteration in their viabilities. A significant correlation between these effects and the titers of both vitellogenin in the haemolymph and vitellin in the ovaries was observed. Ecdysteroid titers in haemolymph and ovaries, as determined by radioimmunoassay, were decreased by this treatment. *In vitro* analysis suggested that azadirachtin may directly interfere in ovarian ecdysteroid production. The significance of these findings in relation to the mode of action of azadirachtin is discussed.

Since the discovery of azadirachtin as a feeding and growth inhibitor, a significant body of literature has accumulated detailing these effects for most insect orders [1–7]. In general this compound disrupts the ecdysteroid-induced moulting process in holometabolous [7, 8] and in hemimetabolous insects [4, 9]. In *Rhodnius prolixus,* low doses of azadirachtin, given orally, cause inhibition of both feeding and moulting [5, 10]. The latter effect can be partially or completely rescued by simultaneous administration of either juvenile hormone or ecdysone, respectively [5]. Ecdysteroid titers in the haemolymph of *Rhodnius* larvae treated with azadirachtin are too low for an induction of ecdysis [11].

In addition to inhibiting feeding, development and ecdysteroid production, azadirachtin may induce sterilization in several species of insects [3, 12, 13]. Azadirachtin suppresses the juvenile hormone [13] and ecdysteroid titers in adult females of *Locusta migratoria* [3].

In this paper, we investigated the effects of azadirachtin A on both oogenesis and vitellogenin synthesis which in *Rhodnius* are dictated by juvenile hormone [14]. Accordingly, we also studied azadirachtin action on *in vivo* and *in vitro* production of ovarian ecdysteroids in adult females of *Rhodnius prolixus*. A preliminary account of some of the work presented in this paper appeared elsewhere [15].

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Materials and Methods

Insects

Rhodnius prolixus were reared and maintained as described elsewhere [16]. Adult females were removed from the stock colony on the day of their last ecdysis and kept separately in glass jars. Virgin females were mated 1–2 days before the first feeding as adults.

Human blood and feeding procedure

Citrated human blood, stored at 4 °C for some hours, was used. Groups of at least 20 females, weighing 65.1 ± 8.5 mg, were starved following the imaginal ecdysis for 15–20 days. They were then allowed to feed for 30 min on human blood by use of a special feeding apparatus [17]. Purified azadirachtin A was diluted in 1:4 ethanol–saline and added to the meal as described previously [5]. Only insects that ingested at least 2.5 times their body weight in one feeding session were used. Control groups received blood and azadirachtin solvent.

Measurement of oocytes

The ovarian growth was determined at various times after feeding and the length of the seven terminal oocytes (T oocytes) of each ovary was measured [18].

Preparation of vitellogenin antiserum

Antiserum to egg proteins of *R. prolixus* was obtained by subcutaneous injection of 1.5 mg of egg

proteins emulsified with Freund's complete adjuvant (Difco) into rabbits. Six weeks later the process was repeated with Freund's incomplete adjuvant. The endovenous booster (with 500 μg of egg proteins) was given two weeks after the second injection. The sera from three rabbits were collected 9 days later and combined. The antiserum titer was monitored by the Ouchterlony immunodiffusion test [19].

Vitellogenin and vitellin determination

The concentration of the haemolymph vitellogenin and vitellin in the ovaries was determined by the Mancini technique [20].

Radioimmunoassay (RIA) for ecdysteroids

For the RIA procedure an ecdysone antiserum (DHS # 1–3.5 wk) binding ecdysone and 20-hydroxyecdysone in the ratio 1:2.6, respectively (generous gift of Dr. J. D. O'Connor, U.S.A.) was used. RIA activity was defined as pg equivalent to 20-hydroxyecdysone since this ecdysteroid was used as the standard.

In vitro ovary assay

Ovaries used for the *in vitro* assay for ecdysteroids were dissected from adult females on day 4 after feeding. After washing in cold *Rhodnius* saline [24] one pair of ovaries was transferred to 100 μl of TC 199 medium (Boehringer Mannheim) and incubated for 4 h at 28 °C under gently shaking. The ecdysteroid content in all groups of ovaries was determined just before incubation. After incubation the medium plus ovaries were collected and prepared for ecdysteroid determination. A linear relationship between the production of ecdysteroid and time of incubation was observed.

Results

Effects of azadirachtin A on oviposition

Four groups of 20 insects each (A–D) received different doses of azadirachtin A. The doses (μg/ml of blood) were: 0.1 μg (B); 1.0 μg (C); 5.0 μg (D); and a control group (A) that received blood with solvent only. Egg production during the first oviposition cycle is shown in Fig. 1. Azadirachtin A drastically decreased egg production in *Rhodnius* at the two higher concentrations (1.0 μg and 5.0 μg/ml of blood; groups C and D). These groups never reached the same rate of egg production attained by the control females (group A). For a period of 24 days after feeding, control females produced an average of 23 eggs. In the azadirachtin-treated groups at the higher doses 7 eggs or even less were produced (Fig. 1). The group B which was treated with 0.1 μg azadirachtin A/ml of blood only, did not present significant difference from the control (group A). The eggs laid by treated females were not apparently affected in their viabilities. The azadirachtin A effect was not reversible by the third oviposition cycle after the females received two fresh blood meals without the compound (data not shown).

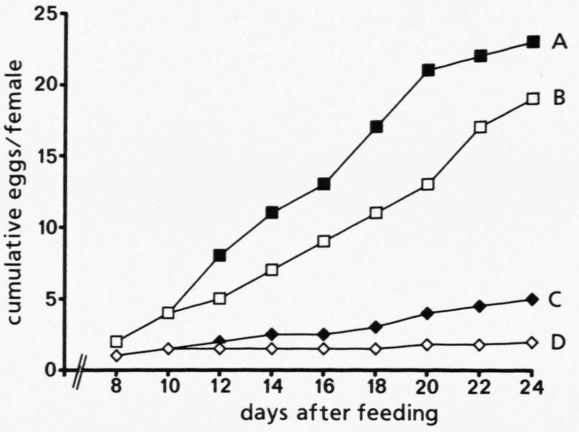

Fig. 1. Cumulative number of eggs laid in the first cycle of oviposition by adult females of *Rhodnius prolixus*. Controls (A); treated with 0.1 (B); 1.0 (C) or 5.0 (D) μg azadirachtin A/ml of blood. Groups of 20 adult females.

Oogenesis in normal and azadirachtin-treated females

Each of the two teletrophic ovaries of *R. prolixus* has seven ovarioles containing oocytes in various stages of development. After feeding and mating, yolk deposition begins and as yolk accumulates the T oocytes enlarge. The effect of azadirachtin A on oogenesis is shown in Table I. The ingestion of 5.0 μg azadirachtin A/ml of blood markedly prevented yolk deposition during the seven days after feeding and the T oocytes showed only a small increase. In the group treated with 0.5 μg azadirachtin A/ml a reduced growth of the T oocytes was recorded.

Table I. Effect of azadirachtin A on the growth of T oocytes, 0–6 days following feeding.

Groups	Days after feeding	Average length of T oocytes [mm]*
Control	0	0.39 ± 0.01
	3	0.91 ± 0.05
	5	1.30 ± 0.08
	7	1.85 ± 0.07
Azadirachtin (0.5 µg/ml)	3	0.85 ± 0.04
	5	1.05 ± 0.06
	7	1.15 ± 0.05
Azadirachtin (5.0 µg/ml)	3	0.45 ± 0.02
	5	0.58 ± 0.04
	7	0.84 ± 0.05

* Each value represents the mean ± S.E.M. of the T oocytes present in both ovaries of 5 females.

Effects of azadirachtin A on vitellogenin and vitellin titers

Blood meal stimulates vitellogenin production by fat bodies in several haematophagous insects [14]. To determine whether azadirachtin A induces sterilization due to the inhibition of yolk protein synthesis and/or its deposition in eggs, we measured vitellogenin and vitellin in the haemolymph and ovaries, respectively. The content of vitellogenin and vitellin rose sharply to a peak of vitellin in the ovaries on day 8 and the vitellogenin in the haemolymph on day 10, both decreasing rapidly later. The females treated with 0.5 µg azadirachtin/ml of blood and 5.0 µl/ml failed to show the normal increase of vitellin in the ovaries. The vitellogenin titers presented a small increase in the haemolymph but they never reached the contents found in the maximum peak of the control groups (Fig. 2).

Azadirachtin A and ecdysteroid production

RIA analysis of ecdysteroid titers in the ovaries and haemolymph indicated that ecdysteroid synthesis is also stimulated by feeding (Fig. 3). Ovarian ecdysteroid contents attained a maximum peak on day 4 after feeding; the amount of ecdysteroid remained higher on day 4 and 5 in the haemolymph. The ingestion of 0.5 µg and 5.0 µg azadirachtin A/ml of blood significantly reduced the ecdysteroid levels in both tissues (Fig. 3).

In vitro analysis of ecdysteroid synthesis can give insights into the mode of action of azadirachtin on mature females of *R. prolixus*. We analyzed the ecdysteroid production by ovaries 4 days after feeding. Table II illustrates the results obtained and compares the production of ecdysteroids of control, group receiving treatment *in vivo* with 1.0 µg azadirachtin A/ml of blood, and ovaries taken from controls and incubated in a culture medium containing 0.5 µg and 5.0 µg azadirachtin A/ml of medium. When the control ovaries were removed 4 days after feeding and analyzed immediately for ecdysteroid content they were found to contain 2520 ± 85 pg ecdysteroid/ovary (n = 5 determinations). However,

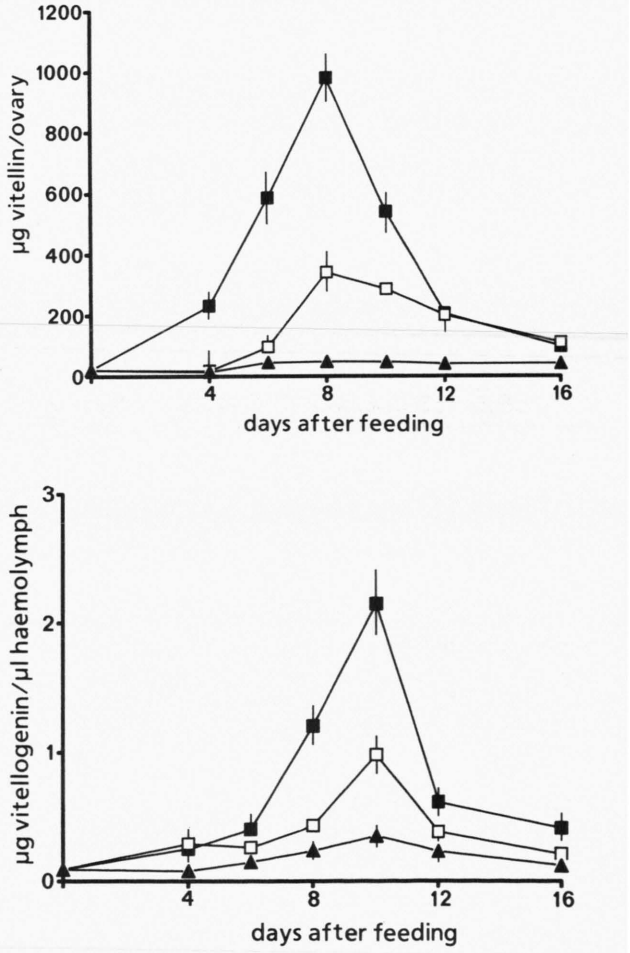

Fig. 2. Effect of azadirachtin A on the haemolymph vitellogenin (below) and ovarian vitellin (above) in adult females of *Rhodnius prolixus*. (■) Controls; (□) treated with 0.5 µg; and (▲) treated with 5.0 µg azadirachtin A/ml of blood meal. Each point is the mean of two individual determinations, except those with vertical bars (± S.E.M.) which were obtained with four determinations.

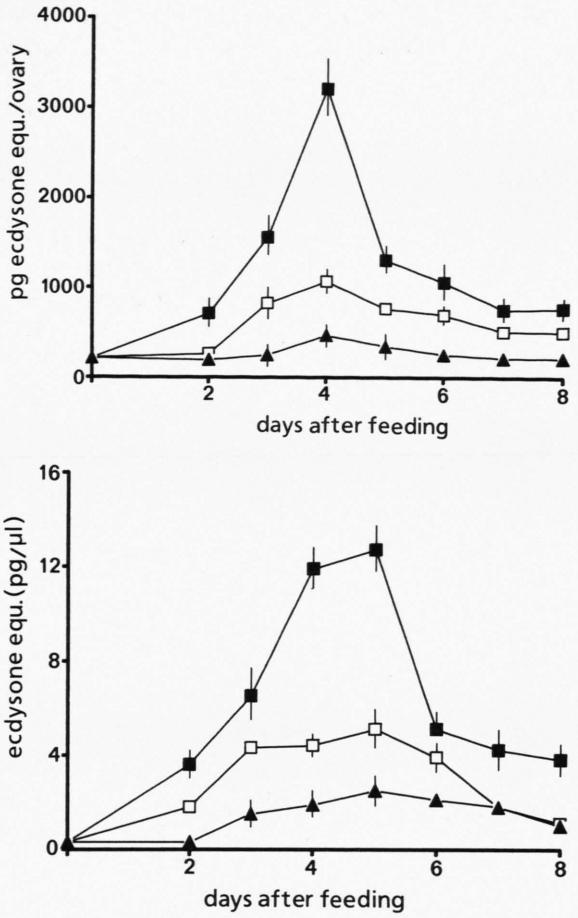

Fig. 3. Effect of azadirachtin A on both ovarian (above) and haemolymph (below) ecdysteroids in adult females of *Rhodnius prolixus*. Details as in Fig. 2.

the incubation of control ovaries significantly increased the total ecdysteroid content (820 ± 105 pg/h/ovary, n = 5 determinations). Ovaries from insects treated *in vivo* with azadirachtin produced little ecdysteroids. While the incubation of control ovaries with 5.0 µg azadirachtin A/ml profoundly reduced ecdysteroid production compared with the control, the ovaries cultured in presence of 0.5 µg/ml had only little inhibition in the production of ecdysteroids.

Discussion

Our results indicate that, in addition to inhibit feeding and moulting in larvae [5, 10, 11], azadirachtin A induces a pronounced sterilization effect in mature females of *Rhodnius prolixus*. We showed that ingestion of azadirachtin A inhibits egg production as measured by oogenesis and egg deposition (Fig. 1 and Table I). This is possibly a consequence of azadirachtin A interference with the synthesis of vitellogenin and/or vitellin deposition into the oocytes, or both. In partial support for this hypothesis we show that both vitellogenin titers in the haemolymph and vitellin contents in the ovaries are markedly reduced in the azadirachtin-treated females (Fig. 2).

In *R. prolixus* it is well known that juvenile hormone controls vitellogenesis [14, 25]. Since azadirachtin depresses the production of juvenile hormone in *Locusta migratoria* [13] we could imagine a similar effect in *Rhodnius*. However, the therapy with juvenile hormone III did not rescue either the

Table II. Spontaneous synthesis of ecdysteroids by ovaries derived from either control or azadirachtin A treatments. The ovaries were removed day 4 after feeding, and they were incubated in TC 199 medium with or without azadirachtin A.

Treatment	Doses of azadirachtin A		pg ecdysteroids production/ovary/h
	µg/ml of blood	µg/ml of medium	
–	–	–	820 ± 105***
*In vivo**	1.0	–	110 ± 22
*In vitro***	–	0.5	610 ± 82
*In vitro***	–	5.0	210 ± 55

* See Materials and Methods.
** Ovaries taken from controls and incubated in culture media containing azadirachtin A.
*** Each number represents the mean ± S.E.M. of 4 individual determinations.

synthesis of vitellogenin or the growth of the ovaries in azadirachtin-treated females [26]. By use of microderivatization and determination of the analyte by combined gas chromatography – selected ion monitoring mass spectrometry, a method which quantifies JH 0–III in the range of 10 femtomoles [27], we analyzed *Rhodnius* haemolymph samples taken from mated adult females from day zero up to day eight after feeding (M. Uhl, unpublished results). Even when one milliliter of haemolymph was analyzed in total, we did not detect any of the four JH homologs. In other words – if any of them would have been present, its concentration would be below 10^{-12} M. This clearly proves that *Rhodnius* does not use the JH 0–III type of hormone molecules and this may be one explanation for the failure of JH III treatment. It seems with this reservation in mind that the effect of azadirachtin on the reproduction of *R. prolixus* females does not relate to the inhibition of juvenile hormone production.

In many insects ecdysteroids play a role in vitellogenesis control [28]. In *Rhodnius* virtually nothing is known about the possible regulation of vitellogenin synthesis by ecdysteroids. However, it has been shown that in adult females the ovaries were required for the appearance of an ecdysteroid peak in the haemolymph [29] and that ovaries accumulated a large amount of ecdysteroid ([26], present paper). Ruegg *et al.* [30] suggested that ovarian ecdysteroids might stimulate the release of the ovulation hormone from the median neurosecretory cells of the brain of mated females. Possibly this neurohormone is involved in ovulation [31]. In contrast, ecdysteroids also decreased egg deposition [18] and reduced activity of the corpus allatum [32, 33]. Therefore, a question arises of a possible relationship between ecdysteroids in adult females and the effect of azadirachtin on reproduction. We showed that azadirachtin limits and reduces the ecdysteroid contents in both haemolymph and ovaries (Fig. 3). Furthermore, we also showed that azadirachtin acts directly on the ovaries decreasing ecdysteroid production (Table II). The relevance of the *in vitro* inhibition of ovaries in secreting hormones compared to the overall effects *in vivo* is often difficult to establish since azadirachtin may act simultaneously on other target systems. On the other hand, the dose of azadirachtin used for *in vitro* experiments may not correspond to the dose utilized for *in vivo* tests, *i.e.*, it is possible that the effects observed for *in vitro* assay is due to a pharmacological and not a physiological dose of the compound. We therefore assume that azadirachtin may interfere in the reproduction by depressing the release of neurohormones and hormones related to vitellogenesis and oogenesis.

Notwithstanding these regulatory considerations, our studies are consonant with the hypothesis that azadirachtin may be used as a tool in a great many questions of a fundamental biological and biochemical nature on the reproduction of adult females of *R. prolixus*. We are currently studying the interaction of this compound in the brain – corpus allatum – ovary interaction, and whether or not it interferes with ecdysone metabolism or the general metabolic pathways in this insect.

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[1] H. Schmutterer and H. Rembold, Z. angew. Ent. **2,** 179–188 (1980).
[2] H. Rembold, G. H. Sharma, Ch. Czoppelt, and H. Schmutterer, J. Plant Dis. Prot. **87,** 290–297 (1980).
[3] H. Rembold and K. P. Sieber, Z. Naturforsch. **36c,** 466–469 (1981).
[4] K. P. Sieber and H. Rembold, J. Insect Physiol. **29,** 523–527 (1983).
[5] E. S. Garcia and H. Rembold, J. Insect Physiol. **30,** 939–941 (1984).
[6] C. P. W. Zebitz, Ent. Exp. & Appl. **35,** 11–16 (1984).
[7] V. Schlüter, H. J. Bidmen, and S. Crewe, J. Insect Physiol. **31,** 773–777 (1985).
[8] R. Steets, Z. ang. Ent. **77,** 306–312 (1975).
[9] A. Dorn, J. M. Rademacher, and F. Sehn, J. Insect Physiol. **32,** 231–238 (1986).
[10] E. S. Garcia, P. Azambuja, H. Forster, and H. Rembold, Z. Naturforsch. **39c,** 1155–1158 (1984).
[11] E. S. Garcia, M. Uhl, and H. Rembold, Z. Naturforsch. **41c,** 771–775 (1986).
[12] O. Koul, Z. ang. Ent. **98,** 221–223 (1984).
[13] H. Rembold, in: Advances in Invertebrate Reproduction, **Vol. 3** (W. Engels *et al.*, eds.), pp. 481–491, Elsevier, Amsterdam 1984.
[14] K. G. Davey, in: Insect Biology in the Future (M. Locke and O. S. Smith, eds.), pp. 325–344, Academic Press, New York 1980.
[15] E. S. Garcia, D. Feder, J. E. P. Lima Gomes, and P. Azambuja, Azadirachtin, a Tool for Studying the Development and Reproduction of *Rhodnius prolixus,* An. Acad. brasil. Ciênc., in press (1988).
[16] E. S. Garcia, P. Azambuja, and V. T. Contreras, in: Genes and Antigens of Parasites, A Laboratory Manual (C. M. Morel, ed.), pp. 43–46, UNDP/World Bank/WHO and Fundacão Oswaldo Cruz, Rio de Janeiro 1984.
[17] E. S. Garcia, M. L. M. Garcia, J. D. Macarini, and F. B. Ubatuba, An. Acad. brasil. Ciênc. **47,** 537–545 (1975).
[18] M. L. M. Garcia, R. P. Mello, and E. S. Garcia, J. Insect Physiol. **25,** 695–700 (1979).
[19] O. Ouchterlony, Acta Pathol. Microbiol. Scand. **26,** 507–515 (1949).
[20] G. Mancini, A. O. Carbonara, and J. F. Heremans, Immunochemistry **2,** 235–241 (1965).
[21] E. S. Chang and J. D. O'Connor, in: Methods of Hormone Radioimmunoassay (B. M. Jaffe and C. J. Behrman, eds.), pp. 797–814, Academic Press, New York 1979.
[22] C. Soumoff, D. H. S. Horn, and J. D. O'Connor, J. Steroid Biochem. **14,** 429–439 (1981).
[23] E. S. Garcia, A. F. Furtado, and P. Azambuja, J. Insect Physiol. **33,** 729–732 (1987).
[24] S. H. P. Maddrell, J. Exp. Biol. **51,** 71–86 (1969).
[25] V. B. Wigglesworth, The Principles of Insect Physiology, 7th ed., Chapman & Hall, London 1972.
[26] E. S. Garcia, D. Feder, J. E. P. Lima Gomes, and P. Azambuja, Mem. Inst. Oswaldo Cruz, in press (1987).
[27] H. Rembold and B. Lackner, J. Chromatogr. **323,** 355–361 (1985).
[28] H. H. Hagedorn, in: Endocrinology of Insects (R. G. H. Downer and H. Laufer, eds.), pp. 271–304, Alan R. Liss, New York 1983.
[29] R. P. Ruegg, F. L. Kriger, K. G. Davey, and C. G. H. Steel, Int. J. Invertebr. Repr. **3,** 357–361 (1981).
[30] R. P. Ruegg, I. Orchard, and K. G. Davey, J. Insect Physiol. **28,** 243–248 (1982).
[31] I. Orchard, R. P. Ruegg, and K. G. Davey, J. Insect Physiol. **29,** 387–391 (1983).
[32] B. Stay, T. Friedel, S. S. Tobe, and E. Mundall, Science **207,** 898–900 (1980).
[33] S. S. Tobe and B. Stay, Am. Zool. **21,** 663–674 (1981).