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HORMONAL CONTROL OF VITELLOGENESIS IN THE IXODID TICK *AMBLYOMMA HEBRAEUM* KOCH

by

Martine Dominique Lunke

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

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2 October 1991

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This note is to confirm that the two submitted papers, comprising the bulk of Martine D. Lunke's M.Sc. thesis, and which are co-authored by me, represent work done for her M.Sc thesis, and I agree to their inclusion in the thesis. Those papers were written by Martine; I contributed significantly to their editing and in other ways as is customary between student and professor.

Reuben Kaufman

UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled HORMONAL CONTROL OF VITELLOGENESIS IN THE IXODID TICK *AMBLYOMMA HEBRAEUM* KOCH submitted by MARTINE D. LUNKE in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in ZOOLOGY.

Dr. W.R. Kaufman, supervisor

Dr. S.K. Malhotra

Dr. R.E. Peter

Dr. R. Ryan

To my late father,
who transmitted to me his scientific curiosity
and his fascination for all living things,

and
to my dear mother.

Abstract

Hormonal control of vitellogenesis in the ixodid tick *Amblyomma hebraeum* Koch (Acari: Ixodidae) was investigated as follows: ovaries from small partially fed or fully engorged females were transplanted into the haemocoel of engorged females that were undergoing vitellogenesis. The transplants appeared to respond to a hormone and began vitellogenesis. The following treatments all failed to induce vitellogenesis within 8 days in small partially fed ticks: (a) slow transfusion of 20-hydroxyecdysone (20-HE; up to 7.2 µg) over 24 hours, (b) topical application or injection of up to 50 µg juvenile hormone III (JH III), (c) topical applications of 10 µg of the JH analogues methylfarnesoate (MF) and JH bisepoxide (JH_{B3}), (d) a combination of 20-HE and JH III. None of the above-mentionned putative hormones accelerated vitellogenesis in engorged females and injections (up to 100 µg) or topical applications (1 mg) of precocene II did not inhibit vitellogenesis in engorged females. This is the first direct demonstration that vitellogenesis in ixodid ticks is under hormonal control, but the data fail to suggest a specific role for an ecdysteroid or JH system.

The effects of the avermectin analogue MK-243 were also tested following injection directly into the haemocoel of engorged females. MK-243 reduced the weight of the total egg mass in a dose dependent way but only slightly increased oviposition latency. Egg laying was almost completely inhibited at 100 µg/kg body weight. MK-243 markedly inhibited ovary development and vitellogenesis

10 days post-engorgement, but its exact mode of action remains unknown: it may have acted either on the synthesis of vitellogenin, on its uptake by the ovary or on the synthesis of the vitellogenic hormone. Ticks treated with 100 µg MK-243/kg also had one-tenth the haemolymph ecdysteroid concentration compared to controls 10 days post-engorgement. Although this shows once more the close correlation existing between haemolymph ecdysteroid concentration and vitellogenesis, the two effects may be independent of each other, 20-HE being unable to stimulate vitellogenesis in this species. Alternatively, 20-HE may partly control vitellogenesis in association with other unknown hormones.

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AVM	avermectin
BSA	bovine serum albumin
b.w.	body weight
DMSO	dimethylsulphoxide
E	ecdysone
GABA	γ-aminobutyric acid
20-HE	20-hydroxyecdysone
HPLC	high pressure liquid chromatography
IVM	Ivermectin
JH	juvenile hormone
JH_{B3}	juvenile hormone bisepoxide
MF	methylfarnesoate
MK-243	4"-epi-methylamino-4"-deoxyavermectin B_1
P II	precocene 2
RIA	radioimmunoassay

CHAPTER I

INTRODUCTION

General biology of ticks

Ticks are parasitic arthropods that feed on the blood of terrestrial vertebrates. They are important vectors of disease for humans and other mammals, being able to transmit a surprising variety of pathogenic agents such as viruses (tick born encephalitis), bacteria (rickettsia of the Rocky mountain spotted fever, spirochetes of Lyme disease), protozoa (*Babesia*, *Theileria*) and *Filaria* (Arthur, 1962; Balashov, 1972). Because of the considerable medical and veterinary impact of ticks, much attention is devoted to tick physiology, in the hope of finding more specific methods of control. A better knowledge of tick endocrinology, for example, might lead to the elaboration of a new generation of acaricides that could disrupt hormone-regulated processes such as moulting, vitellogenesis, oviposition, etc. This would be most useful in light of the increasing resistance worldwide of ticks to traditional organophosphorous pesticides. Mimics of naturally occurring hormones are already successfully used against some insect pests (Sehnal, 1983). This thesis reports my attempt to elucidate the hormonal control of vitellogenesis in the tick *Amblyomma hebraeum*, Koch. *A. hebraeum* is among the largest ixodid ticks available. It is prevalent in South Africa, Mozambique and Tanzania and feeds on a wide variety of

wild and domestic hosts. It transmits the rickettsia *Cowdria ruminantium*, the etiological agent of heartwater fever, to cattle (Nuttall et al., 1926; Arthur, 1962).

Argasid and ixodid ticks

The two main tick families, which comprise a total of 17 genera and over 800 species, are the Argasidae and the Ixodidae (Fig. I-1).

Most argasid species live in enclosed habitats such as nests, burrows or human dwellings and thus remain in close proximity to their hosts. The developmental stages of the argasids are the larva, 2 to 7 nymphal instars and the adult. Each immature instar feeds once and then moults. The bloodmeal lasts from a few minutes to 1 to 2 hours and the amount of blood ingested is relatively low compared to ixodids: their body weight increases 5 to 12-fold. Adult females can feed, detach and lay eggs several times. Mating occurs before or after the bloodmeal. The number of eggs produced during each gonadotrophic cycle varies from 50 to 200.

Most ixodid ticks, on the other hand, have evolved an 'open-field' type of parasitism, which reduces their chances of encountering a suitable host. The biological success of ixodid ticks results from their ability to ingest a very large amount of blood at each feeding and to complete their life cycles with only 3 large bloodmeals, each lasting several days: the large volume ingested each time allows considerable somatic growth from one instar to the next, so that the adult size is reached after only one larval and one nymphal stage.

Phylum :	**Arthropoda**		
Class :	**Arachnida**		
Subclass :	**Acari**		
Order :	**Parasitiformes**		
Suborder:	**Ixodida (=ticks)**		
Superfamily :	**Ixodoidea**		
3 Families :	**Ixodidae**	**Argasidae**	**Nutalliellidae**
Genus : (# of species)	Ixodes (250)	Nothoaspis (1)	Nuttalliella (1)
	Margaropus (3)	Otobius (2)	
	Boophilus (5)	Antricola (4)	
	Rhipicephalus (63)	Argas (140)	
	Anocentor	Ornithodorus (90)	
	Nosomma		
	Dermacentor (31)		
	Amblyomma (100)		
	Hyalomma (21)		
	Haemaphysalis (150)		
	Anomalohimalaya (1)		
	Aponema (26)		

Fig. I-1: Systematic classification of ticks within the phylum Arthropoda, according to Krantz (1978)

All *Ixodinae* (genus *Ixodes*) and most *Amblyomminae* (genera *Amblyomma*, *Dermacentor*, *Haemaphysalis* and *Aponema*) need three different hosts to complete their life cycles, but some species in the genera *Rhipicephalus* and *Hyalomma* for example feed on the same animal as larvae and nymphs, moulting on the host and thus reducing the number of host searches to two. Some *Boophilus* and *Margaropus* species complete their whole life cycle on a single individual, the only free-living stage being the unfed larvae (Balashov, 1972; Diehl, 1982).

The feeding period of adult ixodid females is characterized by 3 phases. During the first 6-24 hours following attachment, the body weight remains more or less constant. A few morphological and physiological changes occur as a result of the transition from free-living to parasitic life. Balashov (1972) named this phase the 'preparatory phase'. It is followed by a 'growth phase' lasting several days during which blood is ingested and used towards integument and organ growth. The third 'expansion phase' starts only in mated females 12 to 36 hours before detachment. Most of the bloodmeal is ingested at that time and the body weight increases very rapidly. Ixodid ticks are able to concentrate their bloodmeal by secreting excess fluid back into the host using their salivary glands (Kaufman and Sauer, 1982). Adult females ingest 3 to 7.5 times their engorged weight in blood. Since the engorged weight can exceed 100 times the unfed weight, the volume of the bloodmeal is unmatched by any other hematophagous arthropod.

Although some oocyte development occurs during the bloodmeal, vitellogenesis occurs mainly after detachment from the host. Females lay a single but very large batch of eggs (up to 20,000 eggs for the larger species) and die. Ticks that are removed from the host when they weigh less than about 10% of their normal engorged weight generally do not oviposit (Balashov, 1972).

Structure of the female genital system in ixodid ticks

The structure and development of the genital system have been described in detail by Balashov (1972) and Diehl (1982). The ovary of ixodid ticks is a single, hollow, elongated organ that can reach several cm in length at full development. Its wall is made of epithelial cells, oogonia and/or oocytes. A longitudinal groove runs along one side of the ovary and the most mature oocytes are found on the opposite side. A basement lamina separates the ovary from the haemocoel. As eggs develop, they protrude into the haemocoel, being connected to the ovary wall only by a few specialized elongated epithelial cells (the funicle cells). Unlike the situation in insects, the oocytes are not surrounded by follicle cells. Both ends of the ovary are connected to long paired oviducts which fuse into an unpaired uterus. The uterus is connected to the ectodermal vagina. After mating, the endospermatophore is stored in the cervical portion of the vagina, which forms a pouch-like seminal receptacle in some ixodid species. As they pass through the vagina, eggs are coated with a protein-rich secretion from the tubular accessory glands and

with a lipid-rich secretion from the lobular accessory glands. Gené's organ coats each egg individually after oviposition with another wax layer in order to complete its waterproofing. Gené's organ is a two- or four-lobed sac that evaginates through a small opening in the camerostomal fold (between the scutum and the capitulum).

Oocyte development and vitellogenesis

Oocytes do not develop synchronously during the gonotrophic cycle of ixodid ticks, so eggs of all the different developmental stages are present at any time, the most immature ones being found near the longitudinal groove. This results in an extended oviposition period that lasts several weeks, and allows the production of a larger number of eggs.

Balashov (1972) divided the development of individual oocytes into 5 stages. Primary oocytes first appear in engorged nymphs and begin the *small cytoplasmic growth phase*, which ends when the meiotic prophase is in diakinesis (stage I). The second stage, or *period of great cytoplasmic growth*, is initiated during the adult bloodmeal. The oocytes grow considerably and start protruding into the haemocoel, being attached to the ovary wall only by their thin stalk of funicle cells. The oocyte cell membrane forms microvilli below the basement lamina, thus increasing the surface area available for subsequent vitellogenin uptake. Numerous Golgi and endoplasmic reticulum areas appear in the cytoplasm. At the end of

this stage, the oocyte possesses all the cell organelles necessary for active protein uptake and the first yolk granules appear in the cytoplasm. Vitellogenesis occurs during Balashov's stages III and IV. Vitellogenin, the main yolk protein, is synthesized by the fat body and probably also by specialized 'presumed vitellogenic cells' in the midgut. The fat body of ixodid females proliferates intensively at the end of the bloodmeal (Obenchain and Oliver, 1973; Coons et al., 1990). In *Dermacentor variabilis*, the 'presumed vitellogenic cells' differentiate from the intestinal epithelium during the bloodmeal in females only (Tarnowski and Coons, 1989). Both the fat body trophocytes and the 'presumed vitellogenic cells' of the midgut possess the cellular organelles necessary for synthesis and export of large amounts of protein, such as an abundant endoplasmic reticulum, Golgi bodies, secretory granules, etc (Tarnowski and Coons, 1989; Coons et al., 1990). Immunological staining confirms that both types of cells contain vitellogenin (Rosell-Davis and Coons, 1989). It is possible that the oocytes also synthesize a small amount of this protein, but this is not well documented (Diehl et al., 1982). Biochemical analysis of the vitellogenin of several species show that they are glyco-lipoproteins bound non-covalently to a haem moiety. The haem derivatives are responsible for the brown coloration of the eggs and may serve to camouflage them on the ground (Diehl, 1982). Glycogen and triglyceride inclusions are also present in the oocyte and are thought to originate from the oocyte itself. Electrophoretic and immunological analysis suggest that the protein portion of vitellogenin is not altered when it is incorporated into the oocyte.

Transport of vitellogenin into the cytoplasm is mediated by coated micropinocytotic vesicles that then fuse together to form multivesicular bodies, which ultimately lose their inner membranes and become large yolk granules. Stage IV corresponds to the deposition of the egg shell, which is produced by the oocyte itself: intracellular vacuoles fuse with the plasmic membrane and release their content under the basement lamina, gradually forming a continuous proteinaceous layer around the egg. The shell however remains permeable to vitellogenin and is able to stretch considerably, as the oocyte continues to grow. Stage V oocytes have detached from the ovary wall and are found either in the ovary lumen or in the oviducts (Balashov, 1972; Diehl, 1982).

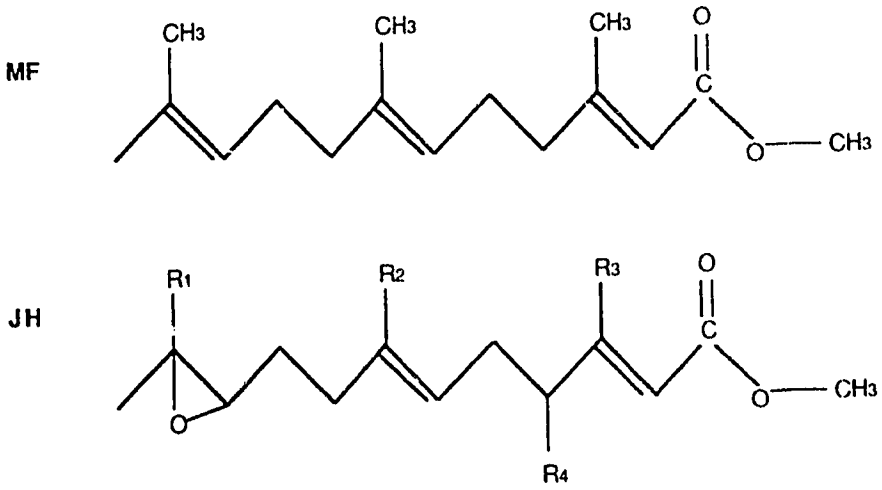
Hormonal control of vitellogenesis in arthropods other than ticks

The presence of well defined endocrine organs and of distinct tagmata have made possible the elucidation of the hormonal control of oogenesis in many insects. The situation in ticks on the other hand is still poorly understood. The cells responsible for hormone synthesis remain unknown and the reduced tagmatosis in the tick body makes the localization of such tissues by ligation experiments virtually impossible. There is some evidence, reviewed later in this chapter, that hormones similar or closely related to those found in insects and crustaceans are present in ticks as well. It is therefore worthwhile briefly reviewing the various mechanisms of endocrine

control of oogenesis in insects, because they offer plausible models for ticks.

Two groups of hormones control vitellogenin synthesis and uptake in insects and perhaps even in all arthropods: the juvenile hormones (JH) and the ecdysteroids. Usually, the two active ecdysteroids involved are ecdysone (E) and 20-hydroxyecdysone (20-HE). Five different JHs have been isolated from insects: JH 0, JH I, 4Me-JH I, JH II and JH III. JH III has been identified in most insect orders but the other forms are found only in *Lepidoptera* (Baker, 1990). Methylfarnesoate (MF), a closely related compound, has been isolated in some crustaceans (Laufer et al., 1987). The chemical structures of these compounds are shown in Fig. I-2.

At least 3 distinct hormonal mechanisms of vitellogenesis regulation exist in insects (Fig. I-3). JH synthesized by the corpora allata control the synthesis and uptake of vitellogenin in most insect species (Engelman, 1983; Postlethwait and Giorgi, 1985). In mosquitos on the other hand, ovarian E, which is converted to 20-HE by peripheral tissues, controls vitellogenin synthesis by the fat body (Hagedorn and Fallon, 1973; Fallon et al., 1974; Hagedorn et al., 1975) but a preexposure to JH is necessary to induce the development of the early ovarian follicles and the maturation of the fat body cells (Gwadz and Spielman, 1973; Flanagan and Hagedorn, 1977; Shapiro and Hagedorn, 1982). Another situation exists in *Drosophila*, in which both the fat body and the follicle cells of the ovary synthesize vitellogenin (Brennan et al., 1982; Hagedorn, 1985): the former can be stimulated in vivo by JH and by E, the latter only by JH (Jowett



	R1	R2	R3	R4
JH O	C_2H_5	C_2H_5	C_2H_5	H
JH I	C_2H_5	C_2H_5	CH_3	H
JH II	C_2H_5	CH_3	CH_3	H
JH III	CH_3	CH_3	CH_3	H
4 Me-JH I	C_2H_5	C_2H_5	CH_3	CH_3

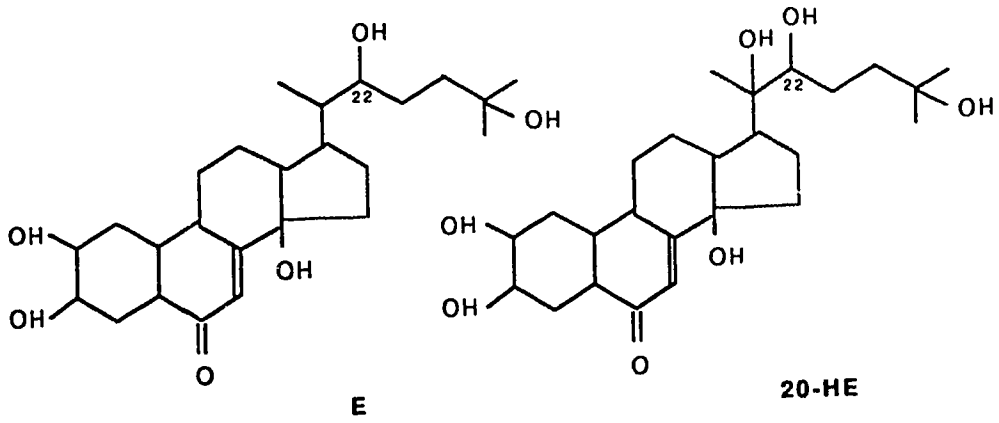


Fig.I-2: Chemical structures of methylfarnesoate, of the insect juvenile hormones JH O, JH I, JH II, JH III and 4Me-JH I and of the two ecdysteroids ecdysone and 20-hydroxyecdysone

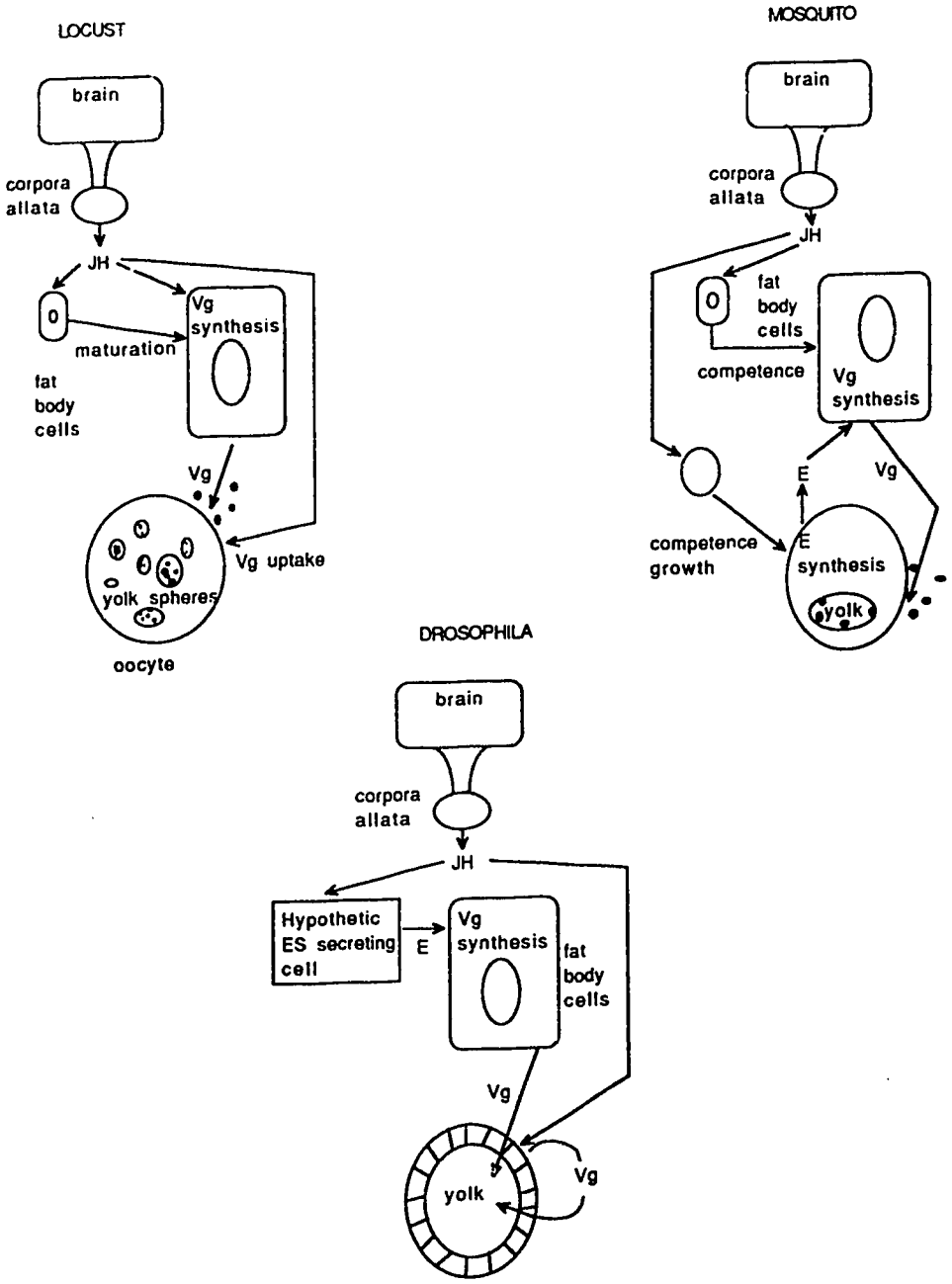


Fig. I-3: Three models for the endocrine control of vitellogenesis in insects (modified after Postlethwait and Giorgi, 1985). JH, juvenile hormone; E, ecdysone; ES, ecdysteroids; Vg, vitellogenin.

and Postlethwait, 1980; Postlethwait and Shirk, 1981) but only JH stimulates vitellogenin uptake by the oocytes (Postlethwait and Handler, 1979). The exact roles of E and JH remain unclear however because it has not been possible to induce vitellogenin synthesis by stimulating vitellogenin-producing cells with these hormones in vitro. Postlethwait and Shirk (1981) have proposed a model in which JH acts indirectly on the fat body by stimulating E release from an unknown source in the abdomen.

Comparatively little is known about the endocrinology of reproduction in arthropods other than insects, but there is some evidence suggesting that JH-like compounds are involved in the regulation of oogenesis in crustaceans and mites. Laufer et al. (1987) for example have shown that the mandibular organs of some crustaceans synthesize MF, which is then released into the haemolymph. In adult females, the highest rate of synthesis coincides with the peak of vitellogenesis, thereby strongly suggesting that this hormone has a function similar to the JH of insects. JH also probably exist in mites because treatment of *Dermanyssus gallinae* with precocene II (P II) greatly reduces the number of offspring of surviving females and this effect is partially reversed by JH III (Oliver et al., 1985).

Endocrine organs of ticks

The tissues responsible for hormone biosynthesis in ticks remain largely unknown. Both JH and ecdysteroids are lipids and are therefore probably synthesized in cells rich in smooth endoplasmic reticulum and lipid vacuoles. The fat body, the ovary and a variety of cells associated with the synganglion are all possible sources of tick hormones.

The fat body of *Amblyomma variegatum* nymphs and of *Dermacentor variabilis* females are reported to release ecdysteroids in the medium when cultured in vitro (Ellis and Obenchain, 1984; Schreifer et al., 1987). It is not clear however if biosynthesis occurs in this tissue or if the hormone is only stored there and then diffuses passively into the incubation medium. Nephrocytes are cells that are closely associated with the trophocytes of the fat body. They also proliferate greatly during the bloodmeal and the preoviposition period in females. Their functions in ticks are not well understood (Obenchain and Oliver, 1973; Coons et al., 1990). They contain smooth endoplasmic reticulum and may therefore be the source of the ecdysteroids released in fat body tissue culture.

Aeschlimann (1968) first demonstrated the importance of the synganglion in the induction of vitellogenesis in argasid ticks. By injecting the homogenized synganglion of fed mated females of *Ornithodorus moubata* into the haemocoel of virgin females, he induced oviposition in 5 out of 12 treated ticks. Injection of virgin female synganglion was ineffective. The gonadotrophic function of a

product from the synganglion was later confirmed with ligation experiments (Shanbaky and Khalil, 1975): when mated *Argas arboreus* females were tightly ligated in the middle of the body immediately after the bloodmeal, ovarian development was blocked only in the rear part of the body which was isolated from the synganglion. This could be reversed by injecting an extract of synganglia from vitellogenic females.

The 'lateral segmental organs' are small groups of cells closely associated with some major nerves of the synganglion. Their ultrastructure suggests that they may be endocrine glands: they contain abundant smooth endoplasmic reticulum and their size and activity increase in fully engorged females of *Boophilus microplus* (Binnington, 1981). The size and activity of the lateral organs also increase after the bloodmeal in larvae of *Argas arboreus*: secretory granules accumulate dramatically just before moulting, when the ecdysteroid levels are the highest (El Shoura, 1989). Is it possible, however, that such small groups of cells can produce the large amount of ecdysteroids circulating in the haemolymph of engorged *Amblyomma hebraeum* females?

Another possible source for ecdysteroids is the ovary itself, which is known to perform such a function in insects (Hagedorn, 1985).

Hormonal control of oogenesis in argasid ticks

Although JH has never been isolated from ticks, there is increasing evidence that a similar or closely related compound may be involved in reproduction of argasid females.

Precocenes have proven very useful to investigate the presence and function of JH in ticks. Precocenes are metabolized intracellularly by JH biosynthetic enzymes into highly reactive epoxides which then destroy the cell. They can therefore be used to inhibit JH synthesis in arthropods in which the site of JH synthesis remains unknown (Bowers, 1983). Po.nd and Oliver (1979) were able to sterilize mated females of *Ornithodorus parkeri* by treating them with P II 24 hours after engorgement. The ovaries of such females showed absolutely no signs of development and remained like those of unfed females. When JH III was applied 24 hours after P II treatment, 7 out of 10 ticks developed vitellogenic eggs in their ovaries. In another study however, although the fertility of *Argas arboreus* females was also considerably reduced after treatment with P II, subsequent JH III treatment failed to restore oogenesis (Leahy and Booth, 1980).

Exogenous JH III can also be used to induce oviposition in some circumstances when it would normally not occur. Treatment of *Argas arboreus* with a JH analogue, for example, terminated diapause and initiated vitellogenesis and oviposition (Bassal and Roshdy, 1974). Also, most unmated fed argasid females do not produce eggs, but topical application of JH or JH analogues on virgin females of *Ornithodorus parkeri* and *Ornithodorus moubata* induced oviposition

in a significant number of these ticks (Obenchain and Mango, 1980; Connat et al., 1983). A similar attempt to induce oviposition in virgin *Argas arboreus* failed however (Bassal and Roshdy, 1974). Obenchain and Mango (1980) also showed that mated females of *Ornithodorus parkeri* fed in vitro on blood containing 20-HE do not lay any eggs, and most of them undergo a supernumerary moult. Treatment of 20-HE fed females with a topical application of JH III however blocked supermoulting and induced oviposition in 6 out of 10 females. All these experiments consistently suggest that JH or a structurally related compound controls ovary maturation in argasid ticks. Moreover, ecdysteroids and JH seem to stimulate bloodmeal utilization in competitive processes: the former stimulating cuticular synthesis for the supernumerary moult and the latter, egg production. Ecdysteroids normally regulate larval and nymphal moults in argasids (Obenchain and Mango, 1980: Diehl et al., 1982; Germond et al., 1982; Connat et al., 1986).

Hormonal control of oogenesis in ixodid ticks

The situation in ixodid ticks remains very confused and the evidence available for JH involvement in oogenesis is only indirect. Hayes and Oliver (1981) fumigated eggs of *Dermacentor variabilis* with a precocene and raised the ticks that hatched. The majority of females that survived to adulthood either could not attach, or attached but did not begin the rapid engorgement phase, or engorged but died soon after. Treatment of these females with JH III enabled

them to engorge completely and to lay eggs. We can however not conclude from these experiments that JH has a gonadotrophic function since it may have stimulated engorgement rather than oogenesis. These results nevertheless suggest that a compound similar to insect JH may exist in ixodid ticks and that it is critical for adult development.

Reduced fertility of females treated with precocenes was reported for *Ripicephalus appendiculatus* and *Boophilus microplus*. No attempts were done to reverse these effects with JH however and the partial sterility reported by these authors could therefore result from general toxicity of the relatively high doses of precocene (Leahy and Booth, 1980; Connat, 1988). Moreover, in these two studies, the sterilizing effect of the precocene was assessed by measuring either the percentage of females that laid eggs which hatched or the total mass of eggs laid. Since precocenes inhibit the proper functioning of Gené's organ (Booth et al., 1986), it is possible that their sterilizing effect is partly attributable to a rapid desiccation of the eggs and not to an inhibition of vitellogenesis.

More convincing evidence for the presence of JH-like compounds during vitellogenesis in ixodid ticks was presented by Connat (1987), who showed that hexane extracts of *Boophilus microplus* females contain a substance that behaves like an insect JH in 2 bioassays, being able to 1) induce oviposition in virgin fed females of *Ornithodorus moubata* and 2) inhibit metamorphosis in *Tenebrio molitor* pupae. When haemolymph extracts were purified by HPLC, several fractions cross-reacted with antibodies directed

against JH I, JH II and JH III. However, the retention time of the immunoreactive products and their comparative affinity to the 3 antibodies suggested that they were neither of the 3 insect JHs. This was confirmed by gas chromatography/mass spectrometry analysis: no JH 0, JH I, JH II, JH III or MF were detected in the samples, but some of the products had spectra compatible with possible structural analogues of JH. All these results suggest that vitellogenic ixodid females contain a substance related to insect JH and necessary for normal ovarian development. The exact nature and function of this hormone remain unknown.

Presence of ecdysteroids on the other hand has been reported in adult females and/or eggs of several ixodid species. The females of *Amblyomma hebraeum* synthesize large amounts of E and 20-HE following the bloodmeal when vitellogenesis is taking place (Connat et al., 1985). The haemolymph ecdysteroid concentration rises from about 9 ng/ml during the first 2 days following engorgement to over 400 ng/ml by days 9-13 (Kaufman, 1991). These ecdysteroids accumulate in free form in the eggs. Their only known function in adult females is to promote the degeneration of the salivary glands that occurs within 4 days after detachment (Harris and Kaufman, 1985; Kaufman, 1991). They do not appear in females that are removed from the host before they reach the 'critical weight' (300 - 500 mg): such females do not show any signs of ovary development and their salivary glands have not degenerated by day 7 post-removal from the host (Kaufman, 1991). It is therefore possible that the release of ecdysteroids (and of the vitellogenic hormone) are

mediated by stretch receptors in the abdomen. Given the close correlation between the titers of ecdysteroids in the haemolymph and the beginning of oocyte growth and vitellogenesis, it has been suggested that these hormones control oogenesis in *Amblyomma hebraeum* (Diehl et al., 1986). A similar situation seems to exist in *Dermacentor variabilis* in which the concentration of ecdysteroids in whole body extracts begins to increase near the end of the bloodmeal and reaches maximum values during oviposition (Dees et al., 1984). The sharp rise in ecdysteroid titer occurs earlier than in *Amblyomma hebraeum*, but vitellogenesis also begins earlier in this species, the first yolk granules appearing in the oocytes during the phase of rapid engorgement (Coons et al., 1989). Thus, once more, there is a close correlation between ecdysteroid synthesis and vitellogenesis. The eggs of *Ripicephalus appendiculatus* also contain E and 20-HE (Whitehead et al., 1986). The pattern of ecdysteroid synthesis appears to be quite different in *Boophilus microplus* however. In this one-host species, larvae attach to the host and detach 21 days later as engorged adults. The titer of free ecdysteroids (E and 20-HE) in whole body extracts peaks one day before detachment from the host. It then decreases to very low values as the ecdysteroids are metabolized to inactive apolar conjugates that accumulate in the eggs (Wigglesworth et al., 1985).

It thus appears that at least 2 different situations exist with respect to the synthesis of ecdysteroids and their storage into the eggs. Whether they reflect different mechanisms of regulating oogenesis remains unknown.

Experimental approach

In order to elucidate the endocrine regulation of vitellogenesis in *Amblyomma hebraeum*, I treated small partially fed females below the critical weight with 20-HE and JH. I assumed that the proper hormone would induce ovary development in ticks at this early feeding stage. I also looked for any effect of exogenous 20-HE, JH or precocenes on vitellogenesis in engorged females.

I was also interested in the effect of avermectins on vitellogenesis in *Amblyomma hebraeum*. Avermectins are anti-parasitic agents that are effective against a broad spectrum of nematodes and arthropods (see Chapter III). Avermectins reduce considerably egg laying if injected into the haemocoel immediately after a bloodmeal (Kaufman et al., 1986). In the second part of this thesis, I tested whether MK-243, an avermectin, has a direct effect on vitellogenesis and on haemolymph ecdysteroid concentration.

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CHAPTER II

HORMONAL CONTROL OF VITELLOGENESIS IN THE IXODID TICK *AMBLYOMMA HEBRAEUM* KOCH

Introduction

Female ticks of the family Ixodidae feed on the host for 7 to 12 days. Their fed to unfed weight ratio can ultimately exceed 100:1. With the exception of the genus *Ixodes*, mating occurs during the bloodmeal and is necessary to stimulate full engorgement. Vitellogenesis then proceeds and oviposition begins within 2 weeks of engorgement (Balashov, 1972). Vitellogenin, the main yolk protein, is synthesized by the fat body and probably also by specialized cells of the midgut (Coons et al., 1982; Diehl et al, 1982; Coons et al., 1989) and is incorporated, supposedly without further modification, into the developing oocytes (Diehl et al., 1982).

The hormonal control of vitellogenesis is still poorly understood in ticks. In most insects, juvenile hormone (JH) stimulates both vitellogenin synthesis and its uptake by the oocytes (Engelmann, 1983). In mosquitoes, JH influences maturation of the fat body and development of the early ovarian follicle, but vitellogenesis itself is

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controlled by ovarian ecdysteroids (Hagedorn, 1985). In *Drosophila*, the situation is also complex, with both JH and an ecdysteroid involved in the control of vitellogenesis (Postlethwait and Shirk, 1981). More recent reports on the possible involvement of JH-like compounds in the reproduction of mites and crustaceans suggest that such hormones may be common to all arthropods (Oliver et al., 1985; Laufer et al., 1987). In argasid ticks, treatment with JH or JH analogues can induce oviposition in virgin fed females of *Ornithodorus moubata* (Connat et al., 1983b), diapausing females of *Argas persicus* (Bassal and Roshdy, 1974) and restore oogenesis in females of *O. parkeri* previously sterilized with precocene (Pound and Oliver, 1979). Evidence for involvement of JH in reproduction in ixodid ticks is indirect and meagre. Topical application of JH III enabled *Dermacentor variabilis* females that had been treated with a precocene as embryos to successfully engorge and to complete their gonadotrophic cycle, but the experiment provided no clues as to where JH was acting (Hayes and Oliver, 1981). Other authors have also reported antigonadotrophic effects of precocenes on ixodid ticks, but they did not demonstrate that these effects could be reversed by application of JH (Leahy and Booth, 1980; Connat, 1988). Also, hexane extracts of vitellogenic *Boophilus microplus* contain substances that are able to 1) inhibit metamorphosis of *Tenebrio molitor* pupae and 2) induce oviposition in fed virgin *Ornithodorus moubata* females. Although these extracts behaved like insect JH, their analysis by mass spectroscopy did not reveal any known insect JH (Connat, 1987).

Presence of ecdysteroids in ixodid ticks, on the other hand, has been reported in females and eggs of several species (Dees et al., 1984; Connat et al., 1985; Wigglesworth et al., 1985; Whitehead et al., 1986; Kaufman, 1991). Females of *Amblyomma hebraeum* synthesize large amounts of ecdysone (E) and 20-hydroxyecdysone (20-HE) following the bloodmeal (Connat et al., 1985) if they feed beyond a 'critical weight' (Kaufman, 1991). These ecdysteroids first appear in the haemolymph and then accumulate in the eggs. Their only known function is to induce the degeneration of the salivary glands (Harris and Kaufman, 1985; Kaufman, 1991), but it has been suggested that they may also control vitellogenesis (Diehl et al., 1986).

In this paper, we report our attempts to probe the vitellogenic hormone in ixodid ticks. We tested whether JH III, JH analogues or 20-HE were able to trigger vitellogenesis in small partially fed females of *A. hebraeum* below the critical weight or if they were able to accelerate vitellogenesis in engorged females. We also tested the effect of precocene II (P II) on vitellogenesis in engorged females. Although we could demonstrate the existence in engorged females of a haemolymph-borne factor which triggers vitellogenesis, we could not reproduce this effect with authentic JH III, two JH analogues (MF and JHB3) nor with 20-HE.

Materials and methods

1) Ticks

All ticks came from a laboratory colony kept in darkness at 27°C, >95% relative humidity. Adult ticks were fed on rabbits as described by Kaufman and Phillips (1973) and removed when they reached the suitable size for each experiment. Small partially fed females below the critical weight weighed between 150 and 290 mg and are hereafter referred to simply as small females. Engorged females were taken less than 24 hours after detachment from the host and weighed between 0.8 - 3.0 g. Ticks were kept in glass vials in the colony incubator between treatment and dissection.

2) Hormones and drugs

The 20-HE was purchased from Simes (Milan). Stock solutions of 1.2 and 12 mg/ml in 95% ethanol were stored at -15°C. The JH III was dissolved in acetone at a concentration of 10 mg/ml and kept at -15°C. The two juvenile hormone analogues methylfarnesoate (MF) and JH III bisepoxide (JH_{B3}) were a gift from Dr. Stephen Tobe, University of Toronto. The entire samples (≈1mg) were dissolved in 200 µl acetone, giving a final concentration of more than 5 µg/µl, and stored at -15°C. P II, from Sigma, was kept in its solid form at -15°C.

3) Injections and topical applications

All ticks were surface sterilized in 70% ethanol. Injections into the haemocoel were done through the soft articulation between the scutum and the capitulum. Infusions over 24 hours with 20-HE were done essentially as described by Harris and Kaufman (1985). The stock solutions were diluted in sterile modified TC199 medium (Gibco; supplemented with 2.09g 3-N-morpholinopropanesulfonic acid (MOPS) and 2.1g NaCl per liter, pH=7.3) to a final concentration of 6 µg/ml or 60 µg/ml. Controls were infused with 0.5% ethanol in modified TC199. These solutions were taken up in sterile 1-ml glass syringes that were then mounted on a Harvard microliter-syringe pump, modified to hold up to 12 syringes simultaneously. The pump was run for at least 15 minutes before mounting the ticks in order to stabilize the delivery rate to approximately 5 µl/hour. The ticks first received a preinjection of 2 µl/100 mg body weight of the infusion solution via a sterile 'Agla' micrometer syringe (Wellcome reagents Ltd.) mounted with a 30g needle, in order to rapidly increase their haemolymph hormone concentration. They were then infused for 24 hours with the microliter syringe-pump and received approximately 0.72 and 7.2 µg/tick (equivalent to about 9 and 90 nmol 20-HE /g body weight respectively for small ticks). Untreated ticks were also kept as additional controls. Ticks were dissected 1, 4 or 8 days after removal from the host.

JH III was applied either topically on the dorsal cuticle in 1 or 2 µl acetone or injected into the haemocoel in 5 µl olive oil. The doses used were 0, 1, 10 or 50 µg/tick. MF and JHB_3 were applied

topically at a dose of 10 µg in 2 µl acetone. Ticks were dissected 1, 4, 8 or 12 days later.

P II was either applied topically (1 mg in 2 µl acetone) or injected (25, 50 or 100 µg in 1 µl dimethylsulphoxide; DMSO) and the ticks were dissected 8 days later

4) Vitellogenesis assay

The ovaries and oviducts were removed, gently blotted, weighed, homogenized in 3.4 ml distilled water and then stored at -15°C. In some of the experiments, the dissected ovaries were first observed under a dissecting microscope in order to evaluate their general stage of development and to measure the length of the largest oocytes with an ocular micrometer. On the day of analysis, the samples were thawed, vortexed, centrifuged at 20,000g for 15 minutes and the supernatant analyzed by spectrophotometry. The difference of absorbance between 400 nm (near the peak absorbance for the haem moiety of vitellin) and 500 nm (non-specific to haem) reflects the total amount of vitellin in the ovary. This difference of absorbance divided by the weight of the ovary reflects the concentration of vitellin in the tissue sample.

5) Assay for salivary fluid secretion

Salivary fluid secretory competence was measured by ligating the main duct of the isolated salivary gland, incubating it for 15 minutes in modified TC199 medium containing 10 µM dopamine, and

measuring net weight increase of the gland. This assay is described in detail by Harris and Kaufman (1984).

6) Collection of haemolymph and preparation of samples for RIA

Sampling of haemolymph was done as described by Kaufman (1991). Briefly, ticks were glued ventrally to a Petri dish and covered with ice for 15 minutes. Incisions through the integument (about 5 mm long) were made along both sides of the tick. The exuding haemolymph was collected with a volumetric capillary tube and added to 150 µl of methanol in a small microfuge tube. The samples were stored in a freezer at -15 °C. Prior to RIA, the samples were vortexed, centrifuged at ≈ 8000 g for 5 min. and the supernatant was collected. The pellet was washed twice with 50 µl of fresh methanol. The combined washes were then evaporated under vacuum and redissolved in an appropriate volume of borate buffer/BSA (0.05 M borate buffer containing 50 mg BSA/ 100 ml, pH= 8.5).

7) Radioimmunoassay (RIA)

This assay was done as described by Kaufman (1990), using 20-HE as standard. The antibody used in this study was directed against ecdysone-22-succinylthyroglobulin and was diluted to 1/2800, giving a B_0 of 25-30%. Since the antibody cross-reacts with several ecdysteroids, data are reported as pg 20-HE equivalents/µl haemolymph. Protein A was prepared according to the method of Kessler (1981).

8) Haemolymph transfusions

Both the donor and the injected tick were surface sterilized with ethanol. The haemolymph of an engorged female undergoing vitellogenesis or ovipositing (from 5 to 16 days after engorgement) was collected as described above, put in a small plastic tube kept on ice, taken up in a 100 µl Hamilton glass syringe and immediately injected into the haemocoel of a small partially fed female freshly removed from the host. Ticks were dissected 10 days later and their ovaries were processed as described under 'Vitellogenesis assay'.

9) Ovary transplants

We transplanted ovaries from small or engorged ticks into the haemocoel of females that had engorged 1 to 4 days earlier.

Both the donor and the recipient tick were surface sterilized in 70% ethanol, and the surgery was done in a sterile air-flow cabinet. The ovary of the donor was dissected out in sterile modified, medium TC199. Recipient ticks were restrained in a vacuum apparatus (described by Harris and Kaufman, 1981) so that the gut diverticula would not protrude nor the gut wall rupture during surgery. Ticks were glued in the apparatus so that only a 3-4 mm square of the dorsal cuticle was exposed. An incision was made along 3 sides of the square and the resulting flap of integument was gently lifted. Vacuum was adjusted so that a space was created between the gut diverticula and the dorsal integument. The ovary of the donor was placed in the haemocoel and gently pushed away from the site of

incision with a fine glass rod. The flap of integument was then repositioned, the vacuum reduced so that the gut diverticula would come in contact with the dorsal integument again and little air would be trapped, and the wound was sealed with cyanoacrylate glue. The operated ticks were dissected 8 to 13 days later, when they started to oviposit. Both the indigenous and the transplanted ovaries were weighed and observed under the dissecting microscope in order to evaluate their general stage of development and to measure their largest oocytes.

10) Statistics

Results are reported as mean +/- SEM (n=). N is also recorded on the graphs above the standard error bars. Differences were analyzed using Student's T-test or a one way analysis of variance (ANOVA), as appropriate.

Results

1) Time course of ovary development after engorgement

Fig. II-1 describes the normal development of the ovary between engorgement and the beginning of oviposition (day 11 to 13). Ovaries were dissected from engorged females 0 to 10 days after detachment from the host. From days 0 to 2, the ovaries contained very little vitellin and accounted for only 0.26 +/- 0.03% of the body weight (n=22). The most developed oocytes at this time were about 100 µm in length; they were usually still elongated, translucent and their nuclei were clearly visible under the dissecting microscope. After day 2, the most mature oocytes began to enlarge. Although they became more spherical, their length did not increase. They appeared whiter and they protruded slightly on the side of the ovary opposite to the longitudinal groove. This corresponds to the 'great cytoplasmic growth' phase described by Balashov (1972).

Obvious signs of vitellogenesis were apparent by day 4. The ovary had enlarged and accounted for 0.68 +/- 0.10 % body weight (n=13). The most mature oocytes appeared very light brown under the microscope. Uptake of vitellogenin was confirmed by the increased absorbance of the ovary homogenates in the spectrophotometric assay (0.112 +/- 0.041, n=13 at day 4 versus 0.013 +/- 0.002, n=22, at days 0-2). The ovaries grew considerably up to the beginning of oviposition: at day 10-11, they accounted for 7.3 +/- 1.3% body weight (n=8). The most mature oocytes had then reached their maximum length (585 +/- 20 µm, n=8).

2) Critical weight for salivary gland degeneration and vitellogenesis

In order to determine the critical weight for vitellogenesis and to see if it corresponds to the previously described critical weight for salivary gland degeneration, ticks were removed from the host at various weights during the feeding cycle and held for 8 days before dissection. Their salivary glands were then assayed for fluid secretory competence and their ovaries for vitellogenesis (Fig. II-2). Salivary fluid secretory rate fell rapidly in ticks between 300-500 mg (Fig.II-2A). Ovary weight and vitellin content were low for ticks weighing less than 400 mg (3.51 +/- 0.40 mg and 0.019 +/- 0.041 respectively, n=41). Beyond that weight, these two parameters increased progressively peaking at 54.13 +/- 7.31 mg, n=13, and 0.424 +/- 0.051, n=13, respectively (Fig. II-2 B and C). Since the critical weight for both salivary gland degeneration and ovary development appeared to be between 300 and 500 mg, we used ticks weighing less than 300 mg in all subsequent experiments involving small females.

3) Ovary transplants

We transplanted ovaries from freshly engorged females into the haemocoel of other engorged females to see if they would continue their development under these experimental conditions. Then, in order to test whether the ovaries of small partially fed females were already competent to respond to a hormone and to begin vitellogenesis when placed in a suitable environment, we

transplanted ovaries of small females into the haemocoel of females that had engorged 1 to 4 days earlier.

Of the 9 ticks that received ovaries from engorged females, 3 died before the beginning of oviposition. The transplanted ovaries degenerated in two of the surviving females, possibly because they had come in contact with the cyanoacrylate glue. In the 4 remaining ticks, the transplanted ovaries had vitellogenic eggs (from a few to half of their surface covered with vitellogenic eggs). In one transplant experiment, the ovary from a female which already showed the very first signs of vitellogenesis, was transplanted into the haemocoel of another vitellogenic female. Ten days later, the transplanted ovary was fully vitellogenic and some of its oocytes had reached their maximum length and were present in the oviducts.
Of the 11 ticks that received ovaries from females below the critical weight, only 4 survived up to the beginning of oviposition. The transplanted ovaries showed clear signs of advanced vitellogenesis in 3 of them (Fig. II-3A), even though the transplanted ovaries weighed much less (9.04 +/- 3.54 mg versus 178.67 +/- 31.21 mg) and contained smaller oocytes than the indigenous ones (0.41 +/- 0.06 mm versus 0.60 +/- 0.02 mm). Fig. II-3B shows that the ovary of a small female 10 days after removal from the host has not begun vitellogenesis.

4) Injection of haemolymph

Haemolymph from vitellogenic or ovipositing females was collected and immediately injected into the haemocoel of small

partially fed ticks to test its effect on vitellogenesis 8 days later. Out of 14 treated ticks, only five survived until dissection: 3 of them had received 10 µl, one 70 µl and the last one 80 µl of fresh haemolymph. Their ovaries showed no signs of vitellogenesis and were typical of females below the critical weight: they weighed 0.86 +/- 0.16% body weight (n=5), the absorbance of the ovary homogenates was only 0.004 +/- 0.001 (n=4) and the length of the largest oocytes was 71 +/- 10 µm (n=4).

5) Infusions with 20-hydroxyecdysone

Small females were infused with a total of 0, 0.72 or 7.2 µg 20-HE/tick (see Materials and Methods) and dissected 8 days post-engorgement. Salivary fluid secretory rate was significantly reduced ($p<0.025$) in ticks receiving 0.72 µg 20-HE over 24 h. Most of the glands from ticks receiving 7.2 µg 20-HE had degenerated completely and could not be dissected out after 8 days (Fig. II-4A). The hormone however did not stimulate ovary growth (Fig. II-4B) nor vitellogenesis (Fig. II-4C). It is of interest that 20-HE seemed to induce a proliferation of the fat body in the haemocoel of some ticks, the effect being especially striking at the higher dose.

Engorged females were also infused with 0 or 7.2 µg 20-HE/ tick and dissected 1 or 4 days post-engorgement to test whether such a treatment might accelerate or amplify vitellogenesis. The haemolymph ecdysteroid concentration was very high 1 to 3 hours following infusion (3.1 +/- 1.2 µg/ml, n=9 ; Fig. II-5A, day 1). This was about 7.5 times the peak concentration found in normal females

9 to 11 days after engorgement (Kaufman, 1991). By day 4, the titer had dropped considerably but was still higher than the untreated controls (185 +/- 43 ng/ml, n=10 versus 70 +/- 30 ng/ml, n=9). Despite the high concentration achieved with the infusion, 20-HE did not accelerate ovary growth and vitellogenesis in engorged females: the ovary weight (Fig. II-5B), the length of the largest oocytes (Fig. II-5C) and the concentration of vitellin in the ovaries (Fig. II-5D) were not significantly different from the controls (ANOVA, $p>0.05$).

6) Treatment with juvenile hormones and juvenile hormone analogues

JH III, applied either topically or by injection, was unable to induce ovary growth (Fig. II-6, A and B) or vitellogenesis (Fig. II-6, C and D). There were no significant or dose-dependent effects with either method. Topical application of JH III (50 µg) seemed to reduce slightly the fluid secretory competence of the salivary glands but the difference was not statistically significant (Fig. II-6E).

Neither of the topically applied JH analogues (JHB3 and MF) induced ovary growth (Fig. II-7A), vitellogenesis (Fig. II-7B) or oocyte growth (Fig. II-7C) and neither affected the haemolymph ecdysteroid titer (Fig. II-7D). JHB3 however reduced the fluid secretory competence of the salivary glands ($p<0.025$; Fig. II-7E).

We also treated freshly engorged females with 10 µg of JH III, JHB3 or MF applied topically, to see if these hormones could accelerate vitellogenesis. Ticks were dissected 1, 4 or 8 days later. The hormones did not stimulate ovary growth (Fig. II-8A),

vitellogenesis (Fig. II-8B) or oocyte growth (Fig. II-8C), and did not alter the haemolymph ecdysteroid concentration (Fig. II-8D).

7) Treatment of small females with JH III and ecdysone

In order to test whether a combination of both hormones is required to induce vitellogenesis, small partially fed females were treated with 1 or 10 µg of JH III, either by injection or by topical application, and were then infused with a solution containing a total of 0.72 or 7.2 µg 20-hydroxyecdysone. Ticks were dissected 8 days later.

Ovary growth and vitellogenin uptake were not stimulated in any of the 8 treatment groups (Table II-1). The ovaries of treated ticks were typical of those of small partially fed females 8 days after removal from the host.

8) Treatment of engorged females with P II

P II, applied topically on engorged females, did not inhibit vitellogenesis significantly. The weight of the ovary (Fig. II-9A), its content in vitellin (Fig. II-9B) and the length of the largest oocytes (Fig. II-9C) were not significantly different from the controls.
An earlier attempt to inject 0-100 µg P II in 1 µl DMSO into the haemocoel resulted in a high mortality rate (up to 40%), which may have been due to the toxicity of the DMSO. In the surviving ticks however, there was no significant inhibition of ovary growth or vitellogenesis (ANOVA, $p>0.05$) but the sample sizes were quite small (5-10) because of the high mortality rate.

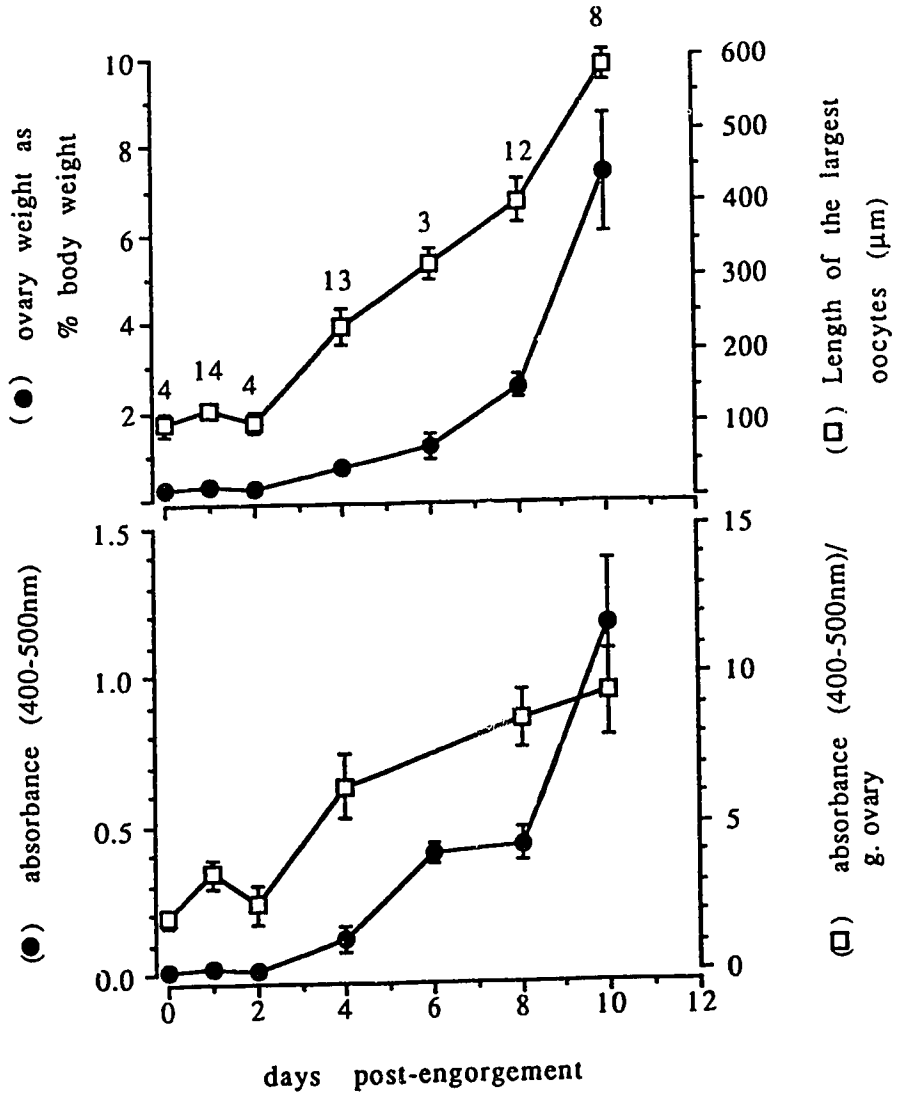

Figure II-1:
Time course of ovary development. The wet weight of the ovary, the length of the largest oocytes, the total amount of vitellin in the ovary (as assessed by the absorbance (400-500nm) of ovary homogenates) and the concentration of vitellin in the ovaries (absorbance (400-500nm) per gram ovary) are shown as a function of time post-engorgement. The SEM is shown wherever it exceeds the dimension of the symbol. N are shown above the symbols and are the same for all 4 graphs.

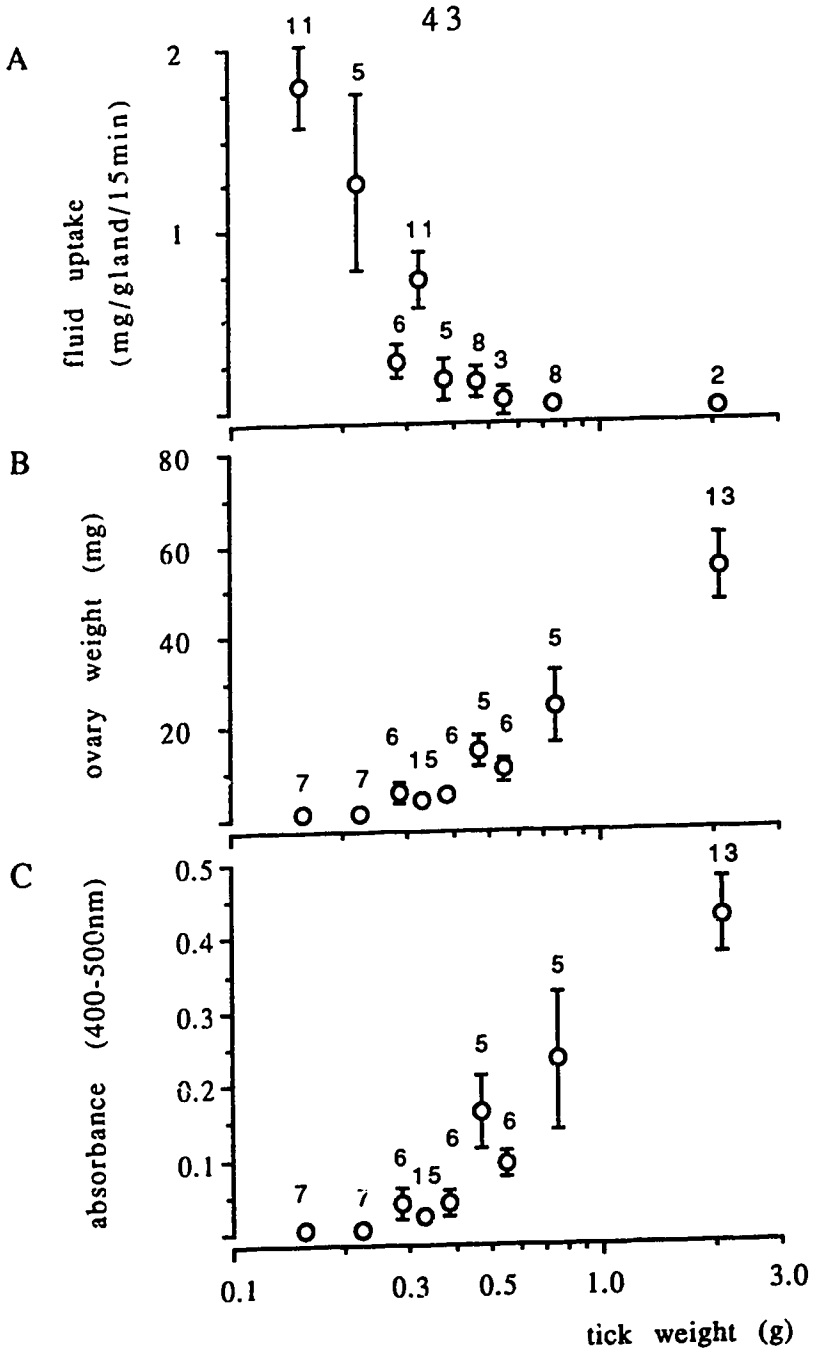

Figure II-2:
Determination of the critical weight for vitellogenesis.
A) Fluid secretory competence of salivary glands; B) ovary weight and C) ovary vitellin content, 8 days after removal from the host or engorgement. Ticks were pooled as follows: tick weight up to 200 mg (157 +/- 9 mg, n=7); 200-250 mg (223 +/- 7 mg, n=7); 250-300 mg (285 +/- 4 mg, n=6); 300-350 mg (327 +/- 4 mg, n=15); 350-400 mg (383 +/- 6 mg, n=6); 400-500 mg (466 +/- 16 mg, n=5); 500-600 mg (550 +/- 10 mg, n=6); 600-1000 mg (752 +/- 35 mg, n=5); and over 1000 mg (2,086 +/- 174 mg, n=13).

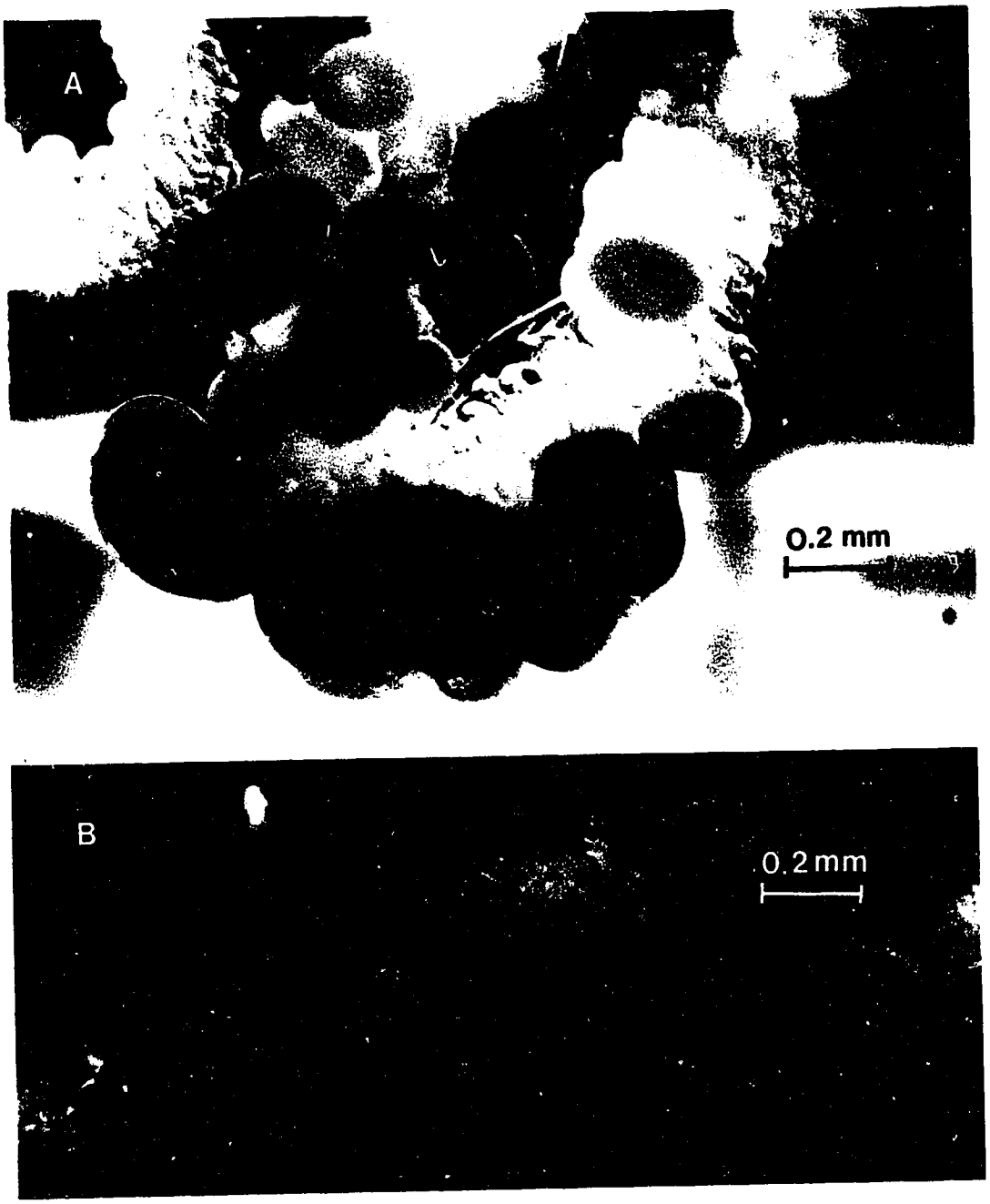

Figure II-3:
A) The ovary of a small partially fed female,transplanted into the haemocoel of an engorged female and dissected 13 days later, contains some vitellogenic oocytes. Magnification: 80 X.
B) The ovary of a female below the critical weight, dissected 10 days after removal of the tick from the host, shows no signs of oocyte growth or vitellogenesis. Magnification: 80 X.

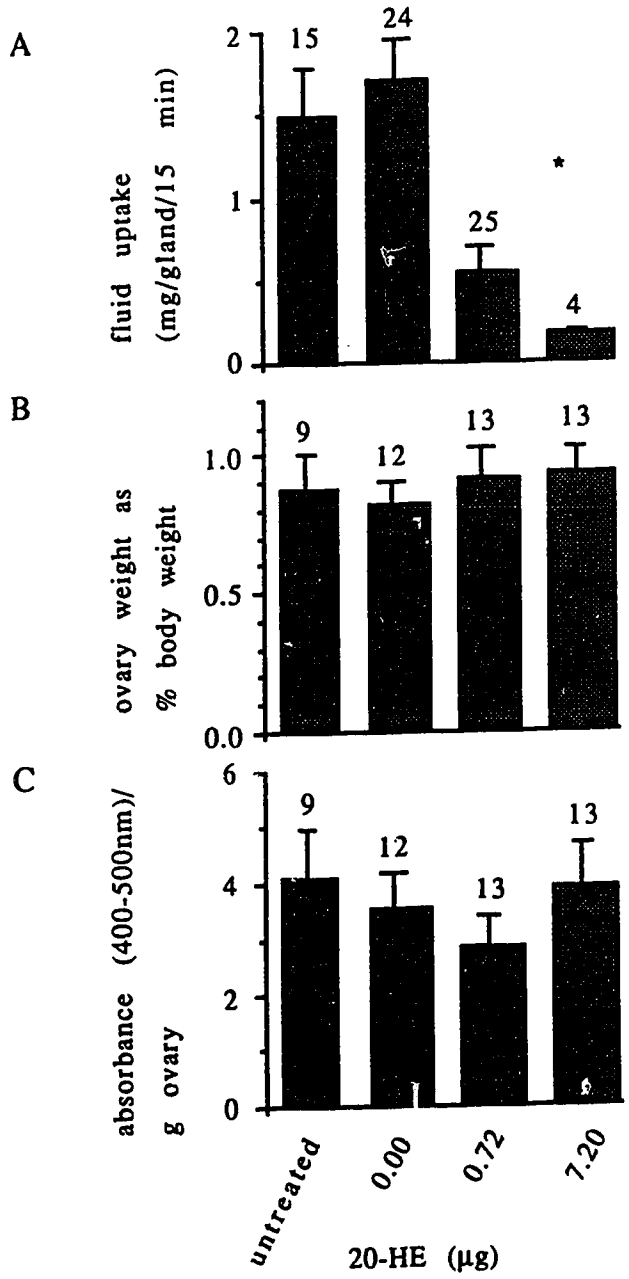

Figure II-4:
Effect of 20-HE on small partially fed females 8 days after removal from the host. A) salivary fluid secretory competence, B) ovary weight as % body weight and C) concentration of vitellin in the ovary. N is shown above the SEM bars and (*) indicates a statistically significant difference (ANOVA, $p<0.025$).

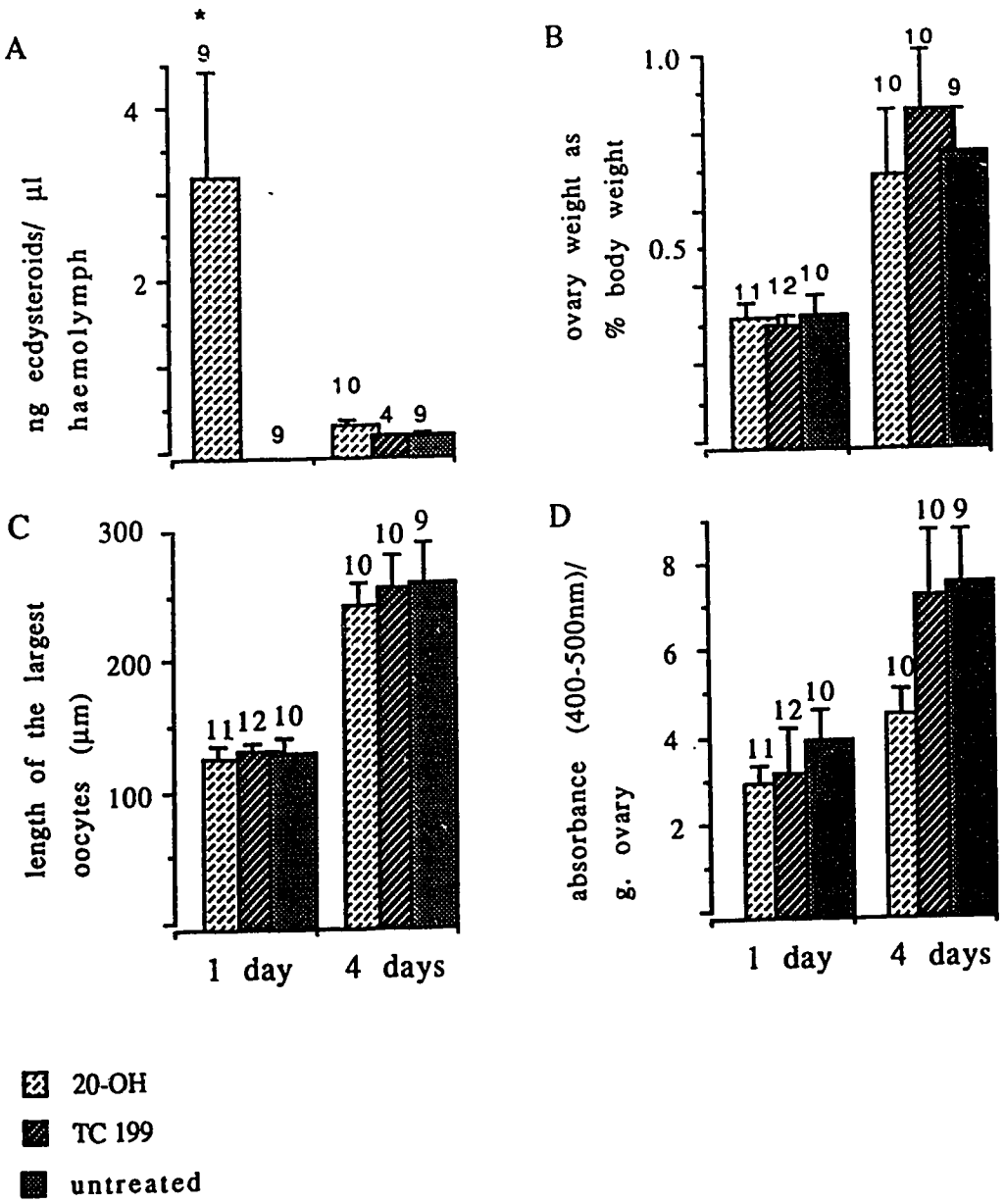

Figure II-5:
Effect of 7.2 µg of 20-HE on engorged females 1 or 4 days post-engorgement. A) Haemolymph ecdysteroid concentration, B) Ovary weight as a % of the body weight, C) Length of the largest oocytes and D) Concentration of vitellin in the ovaries. (*) indicates a significant difference from the controls (T-test, $p<0.025$). Otherwise, differences were not statistically significant (ANOVA, $p>0.05$).

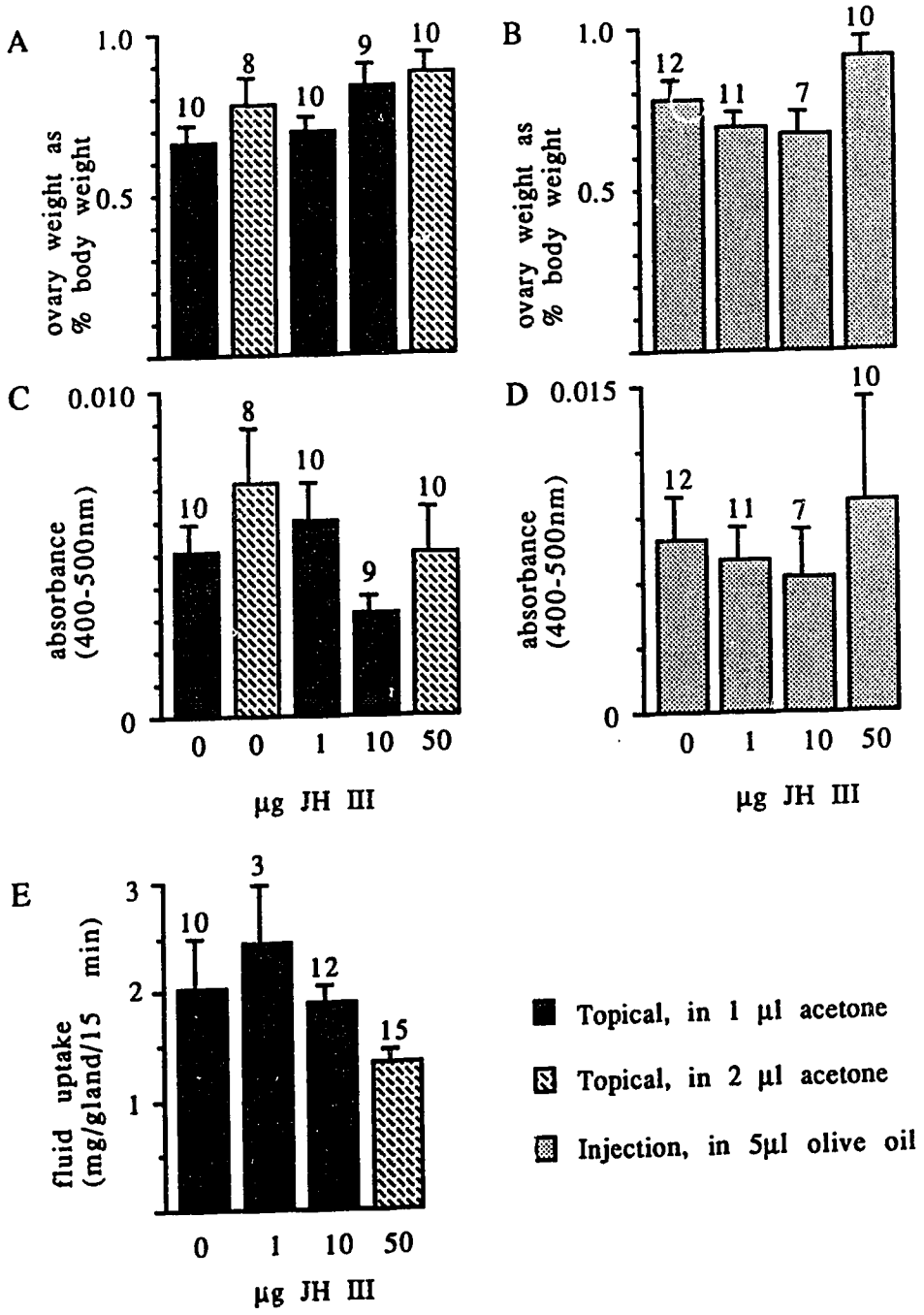

Figure II-6:
Effect of JH III on small partially fed females 8 days post-treatment. A) and B) ovary weight as % body weight, C) and D) total vitellin content of the ovaries, E) salivary fluid secretory competence. Differences were not statistically significant (ANOVA, $p>0.05$).

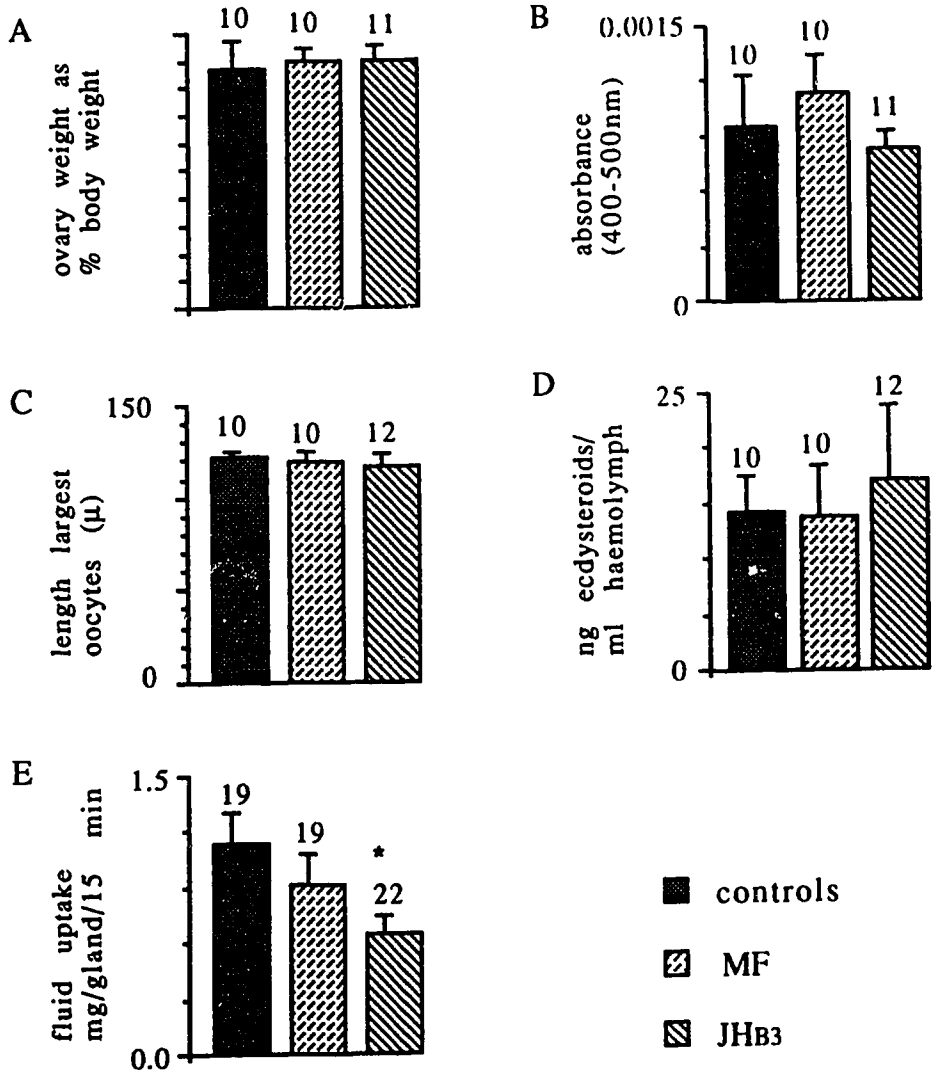

Figure II-7:
Effect of 10 µg of MF or JHB3 on small partially fed females 12 days post-treatment. A) Ovary weight as % of the body weight, B) Total vitellin content of the ovary, C) Length of the largest oocytes, D) Haemolymph ecdysteroid concentration and E) Salivary fluid secretory competence. N is indicated above the SEM and (*) indicates a statistically significant difference with the control (T-test, $p<0.025$).

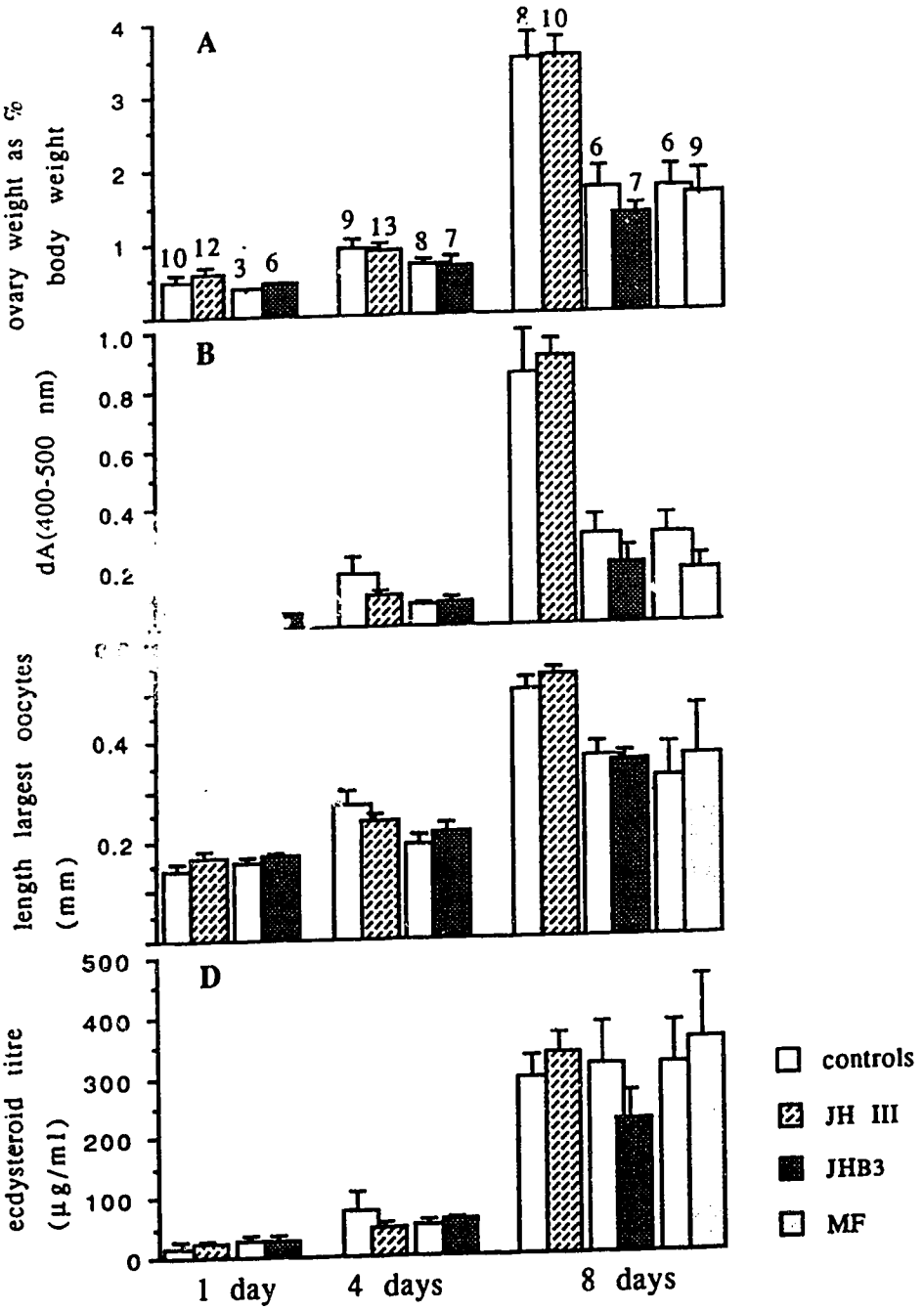

Figure II-8:
Effect of 10 µg of JH III, JHB3 or MF on engorged females 1,4 or 8 days post-treatment. A) Ovary weight as % body weight, B) Total amount of vitellin in the ovary, C) Length of the largest oocytes and D) Haemolymph ecdysteroid concentration. N are indicated above the SEM and are the same for all 4 graphs.

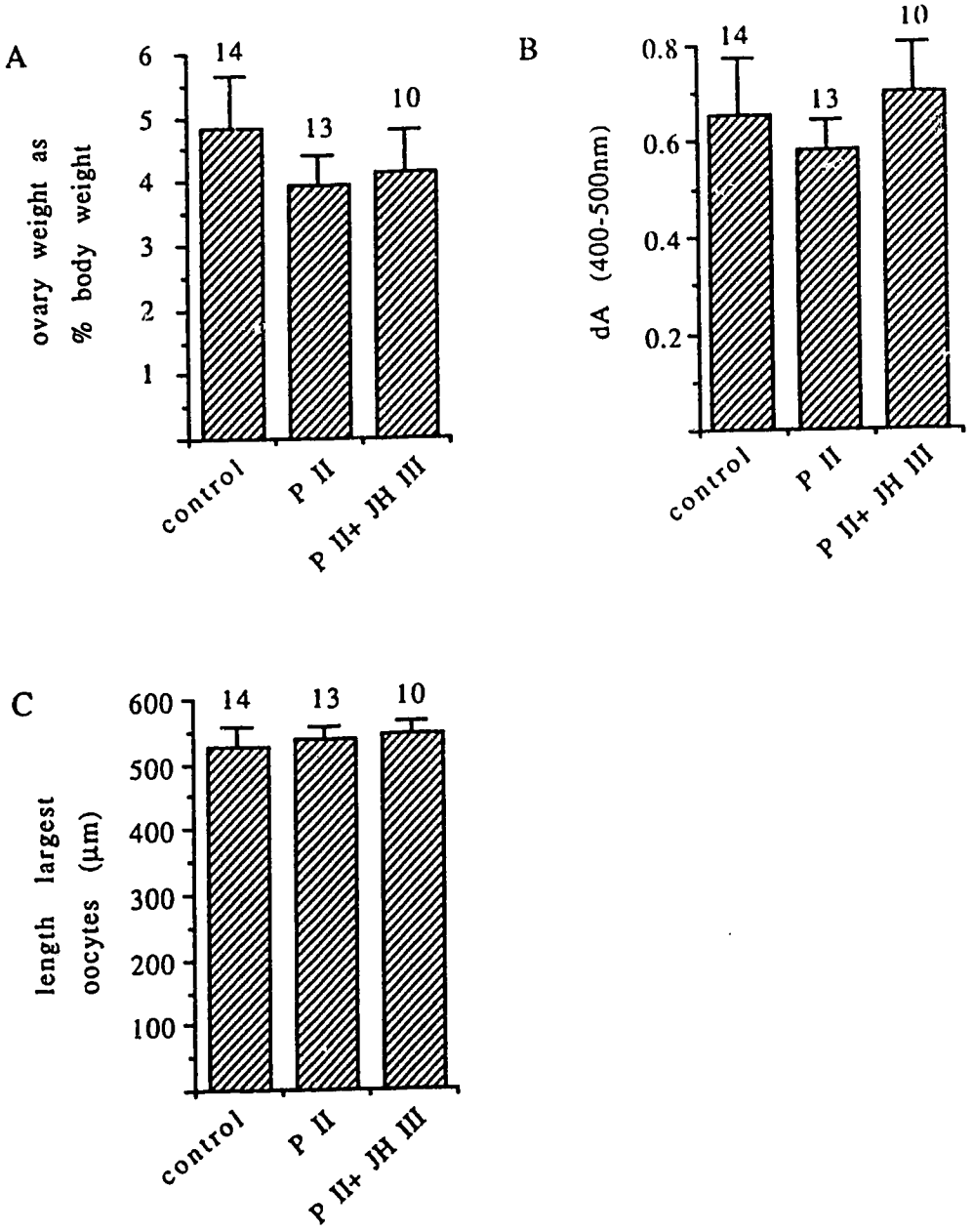

Figure II-9:
Effect of P II and JH III on engorged females A) ovary weight as % body weight, B) Total amount of vitellin in the ovary and C) Length of the largest oocytes, 8 days post- treatment. Ticks were treated topically with acetone (control), 1 mg P II, or 1 mg P II and 10 µg JH III. N is indicated above the SEM. Differences were not statistically significant.

Table II-1:
Indices of ovary development after 8 days following a variety of treatments with 20-HE and JH III. Mortality in these experiments was high: about 50% for injected ticks, 30% for topical applications and 0% for untreated controls. The numbers indicated here pertain only to the ticks which survived for 8 days.

Treatment groups:			n	ovary weight as % body weight	Total vitellin: absorbance (400-500nm)
1) Untreated females below the critical weight (day 8)			10	0.87 +/- 0.11	0.008 +/- 0.003
2) Untreated engorged females (day 8)			12	2.52 +/- 0.26	0.430 +/- 0.055
3) Females below critical weight treated with 20-HE and JH III *:	JH III (µg)	20-HE (µg)			
topical	1	0.72	9	0.90 +/- 0.10	0.006 +/- 0.001
	10	0.72	6	0.94 +/- 0.05	0.006 +/- 0.001
	1	7.2	5	0.90 +/- 0.07	0.006 +/- 0.001
	10	7.2	10	0.96 +/- 0.06	0.008 +/- 0.001
injected	1	0.72	8	0.86 +/- 0.04	0.010 +/- 0.002
	10	0.72	11	0.84 +/- 0.09	0.005 +/- 0.001
	1	7.2	7	0.97 +/- 0.06	0.008 +/- 0.001
	10	7.2	8	0.79 +/- 0.08	0.010 +/- 0.001

* None of the hormonal treatments resulted in an increase in ovary weight or total vitellin above that of the untreated females below the critical weight.

Discussion

Ovaries of small females are able to undergo vitellogenesis when transplanted into the haemocoel of vitellogenic females. This clearly demonstrates that ovary development and vitellogenesis are initiated by a haemolymph-borne factor, which is probably the vitellogenic hormone of ixodid ticks. Ecdysteroids and JH analogues were not capable of mimicking this factor under our experimental conditions.

We cannot rule out the possibility that this factor might be vitellogenin itself and that the ovary does not require hormonal stimulation to undergo vitellogenesis, but this seems unlikely for several reasons. First, the most mature oocytes of partially fed *Amblyomma hebraeum* females are still very small, elongated and translucent. They grow considerably before they begin to accumulate vitellin (see Fig. II-2) and it seems unlikely that vitellogenin is able to initiate the 'great cytoplasmic growth' phase of the oocytes. Secondly, several studies have shown that vitellogenin is not able to stimulate its own uptake in insects. Injection of vitellin into allectomized females of the cockroach *Periplaneta americana*, for example, does not induce vitellogenesis (Bell, 1969). Moreover, Giorgi (1979) showed that JH causes isolated previtellogenic ovaries of *Drosophila* to form microvilli and coated pits, the structural correlates to vitellogenin uptake. It is therefore most likely that there is a vitellogenic hormone present in the haemolymph of engorged female ticks. This is, to our knowledge, the first direct

demonstration of the existence of a vitellogenic hormone in ixodid ticks. Since the ovaries of small females were also able to respond to this hormone, it should have been possible to induce vitellogenesis or at least previtellogenic growth by injecting the proper hormone into their haemocoel.

Transfer of haemolymph from vitellogenic females into small females did not induce vitellogenesis despite the high volume injected in some cases. It is possible that this failure results from a rapid metabolization of the vitellogenic hormone in ticks at this stage. Other studies allude to rapid elimination of hormones injected into the haemolymph (see review by Koolman and Karlson, 1985): 20-HE for example is rapidly metabolized when injected into non-blood fed mosquitos (Fallon et al., 1974) but the half life of the hormone increases from 1 to 8 hours if injected after a bloodmeal (Hagedorn, 1983). Similarly, single or double bolus injections of 20-HE are relatively ineffective to induce salivary gland degeneration in *Amblyomma hebraeum*, a continuous infusion over 24 hours being far more effective (Harris and Kaufman, 1985). The high mortality of ticks in this experiment may have resulted from toxins formed by oxidation of the haemolymph or from bacterial contamination.

20-HE was the first hormone we tested as possible vitellogenic hormone because its concentration in the haemolymph is known to rise after engorgement and to reach a maximum at the beginning of oviposition (Connat et al., 1985). Moreover, the haemolymph concentration of this ecdysteroid remains low in small females that are removed from the host before they reach the critical weight

(Kaufman, 1991). Such ticks show no signs of ovary development or vitellogenesis 8 days after removal (see Fig. II-2). In addition, 20-HE is the vitellogenic hormone of mosquitos (Hagedorn, 1985) and stimulates vitellogenin synthesis by the fat body in *Drosophila* (Postlethwait and Shirk, 1981). In our study, although the final concentration of 20-HE in the haemolymph was well above the level needed to induce complete salivary gland degeneration, 20-HE failed to stimulate ovarian development and vitellogenesis.

JH controls vitellogenin synthesis and uptake in most insects (Engelmann, 1983) and there is increasing evidence that it may be involved in oogenesis in mites and in argasid ticks as well (Pound and Oliver, 1979; Oliver et al., 1985; Connat et al., 1986). MF, a JH-like compound has also been isolated from vitellogenic females of the crustacean *Libinia emarginata* (Laufer et al., 1987). Despite the meagre evidence for the occurrence of JH in ixodid ticks, we decided to test its effects on oogenesis because of the wide phylogenetic distribution of JH-like compounds in arthropods. We chose to use JH III rather than another natural insect JH isomer because it is the most commonly found JH in insects (Baker, 1990) and because it had been used successfully in other experiments involving argasid and ixodid ticks. The doses we used were well within the range of those used in other studies. For example, topical application of 1 µg JH III on females of *Ornithodorus parkeri* previously sterilized with a precocene was sufficient to restore oviposition, 10 µg proved less effective and 100 µg was toxic and did not reverse the effect of the precocene (Pound and Oliver, 1979). Two applications of 1 µg

enabled females of *Dermacentor variabilis* that had been treated with a precocene as embryos to engorge and complete their gonadotropic cycle (Hayes and Oliver, 1981) and 10 µg JH III induced oviposition in some fed virgin females of *Ornithodoros porcinus porcinus* (Oberlain and Mango, 1980). Given the large size and thick integument of *Amblyomma hebraeum*, we used doses of the hormone ranging from 1 to 50 µg JH III. Since we do not know how much of the hormone actually passes through the integument after topical application, we also injected JH III directly into the haemocoel. JH III, JHB3 and MF all failed to induce ovary growth or vitellogenesis in small females. A slight reduction of salivary fluid secretion was measured after treatment with JHB3, but the significance of this observation remains unclear. Shelby et al. (1989) have reported that topical treatment with JH analogues activated the salivary glands of feeding virgin *Amblyomma americanum* females to a level normally seen only in mated females in later feeding stages: salivary gland protein levels and Na/K-ATPase activity were stimulated and morphological changes occured that are normally seen only in the rapid engorgement phase of mated females. This seems to be in contradiction with our results: if JH analogues activate the salivary glands of females during the bloodmeal, one would expect to see an increase rather than a decrease in their salivary fluid secretory competence. The JH analogues did not alter the haemolymph ecdysteroid concentration at day 8, although they may have caused a transient increase of ecdysteroid titers earlier on, which could account for the lower salivary fluid secretion. JH

stimulation of ecdysone synthesis by the prothoracic gland has been reported in some lepidopterans (Hiruma et al., 1978; Hagedorn, 1985) and Postlethwait and Shirk (1981) proposed a model for vitellogenesis control in *Drosophila* in which JH is thought to stimulate ecdysteroid secretion.

We finally also tested the hypothesis that both JH and 20-HE were needed together to induce vitellogenesis, since both groups of hormones are involved in oogenesis in *Drosophila* and in mosquitos. None of our treatments stimulated ovary growth or vitellogenesis (Table II-1).

In all the experiments described above, small ticks below the critical weight were used, and it is possible that at this early stage, the fat body is not competent to respond to the hormone because the fat body cells, for example, could lack the receptors for the hormones. The haemolymph may also contain enzymes that rapidly metabolize the exogenous hormones. We therefore decided to apply the same hormones to freshly engorged females to see if they could accelerate or amplify vitellogenesis. We also treated engorged ticks with P II to see if it could inhibit or slow down vitellogenesis. Although infusion with 20-HE resulted in an initially very high titer of the hormone in the haemolymph (Fig. II-5A), it did not stimulate ovary or oocyte growth, and even may have slowed down the uptake of vitellin, although the difference was not statistically significant (Fig. II-5D). This effect is quite surprising however, because vitellogenesis in *A. hebraeum* normally occurs when the haemolymph concentration of free ecdysteroids is high. The

inhibition we observed could possibly be attributed to receptor down-regulation or to pharmacological or toxic effects, the concentration of 20-HE at the end of the infusion being much higher than the normal physiological concentration. Interestingly, anti-gonadotrophic effects of exogenous ecdysteroids have been reported in several argasid and two ixodid species. Engorged females of *Boophilus microplus* show high mortality and severe inhibition of egg production after injection of 120 ng of 20-HE (Mansingh and Rawlins, 1977). It should be noted however that in this species, the situation is quite different with regard to the ecdysteroids present during vitellogenesis: the concentration of free ecdysteroids in whole body extracts peaks before detachment from the host, but then remains quite low throughout the preoviposition period (Wigglesworth et al., 1985). Topical application of 20 µg of 20-HE one day after attachment also reduced considerably the fecundity and egg hatch of *Hyalomma dromedarii* (Khalil et al., 1984). In this case however, the effect of the hormone may have been indirect: 20-HE was applied at the beginning of the bloodmeal and may have disrupted feeding rather than reproduction. It may also have disrupted the normal osmoregulatory function of the salivary glands, thereby reducing the amount of fluid the tick was able to secrete back into the host, and consequently limiting the amount of blood ingested. (Fecundity in ticks is proportional to the size of the bloodmeal; Balashov, 1972). In the argasid tick, *Ornithodorus moubata*, injection of 50 or 100 ng ecdysone into the haemocoel of vitellogenic females 8 days after the blood meal induced resorption of the eggs, and when females were

fed with blood containing ecdysteroids, the gonadotrophic cycle was inhibited and a supernumerary moult was induced (Connat et al., 1983a).

Topical applications of 10 µg of JH III, MF or JHB_3 also failed to accelerate vitellogenesis, oocyte or ovary growth in engorged females (Fig. II-8).

Finally we tried to treat engorged females with P II, the well-known inhibitor of JH synthesis, but we did not observe any significant effects on ovary growth or vitellogenesis (Fig. II-9). Although it is possible that the concentration of P II reaching the haemolymph after a topical application on the thick dorsal cuticle of *A. hebraeum* is too low to inhibit JH synthesis totally, injection of the precocene into the haemocoel was not more effective (page 41). Our methods of treatment and the dosages we used were, however, similar to those used successfully in other studies: topical application of 500 µg of P II for example sterilized completely *Ornithodorus parkeri* females (Pound and Oliver, 1979) and inhibited oviposition in a significant proportion of *Boophilus microplus* females (Connat, 1988) whereas 1 mg P II considerably reduced the fecundity of *Argas arboreus* females (Leahy and Booth, 1980).

In conclusion, although our experiments clearly show that a humoral factor controls ovary maturation in *Amblyomma hebraeum*, we were unable to demonstrate a definitive role for 20-HE or for JH. We cannot, however, exclude the involvement of JH for several reasons. Although we used doses of JH in the range of those used successfully by other authors, we still do not know how much of our

JH actually reached its target tissue. Moreover, the chemical structure of tick JH, if it exists, may be different from insect JH, and perhaps we did not test the closest analogues. Connat (1987) already suggested a different chemical structure for the JH of *Boophilus microplus*, after a mass spectroscopy analysis failed to detect any known JH in haemolymph extracts that showed JH activity in two bioassays. We know on the other hand that we elevated the haemolymph ecdysteroid concentration considerably in both small and engorged females, but that this did not stimulate ovary development. It therefore seems unlikely that 20-HE controls the entire process of vitellogenesis in this species. 20-HE however seemed to induce proliferation of the fat body in small ticks. Most of the fat body proliferation normally occurs in engorged females during the preoviposition period (Obenchain and Oliver, 1973; Coons et al., 1990). It is therefore possible that a role of ecdysteroids in engorged females is to stimulate maturation and proliferation of the fat body, and perhaps also synthesis of vitellogenin, but that another factor is necessary to stimulate uptake by the oocytes.

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CHAPTER III

EFFECTS OF THE AVERMECTIN ANALOGUE MK-243 ON VITELLOGENESIS AND REPRODUCTION IN THE IXODID TICK *AMBLYOMMA HEBRAEUM*

Introduction

The avermectins (AVMs) are a family of broad spectrum anti-parasitic agents. The analogue ivermectin (IVM) is particularly effective against a broad range of parasitic nematodes and arthropods at very low doses (Campbell et al., 1983; Wright, 1986; Strong and Brown, 1987; Jackson, 1989). Several studies suggest that AVMs act by stimulating γ-aminobutyric acid (GABA) systems. For example, in the ventral nerve cord of the nematode *Ascaris suum*, AVM blocks signal transmission from interneurons to excitatory motoneurons. This effect is reversed by the GABA antagonist picrotoxin (Kass et al., 1980). In the neuromuscular junction of the lobster walking leg, AVM inhibits excitatory and inhibitory postsynaptic potentials by reducing muscle membrane resistance. Since GABA regulates the opening of chloride channels in crustacean

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muscles and picrotoxin can reverse this AVM-induced effect, it is likely that here also AVM stimulates a GABA system (Fritz et al., 1979). AVM can also inhibit chitin metabolism (Calcott and Fatig III, 1984) and causes histopathological changes on the ovaries of fire ant queens (Glancey et al., 1982).

Treatment of the host with IVM neither prevents ixodid ticks from attaching, nor does it cause prompt detachment of those already feeding, but several studies report an interruption of the feeding process. In some cases, ticks remain attached for long periods of time without being able to engorge and they eventually detach or die on the host. Adult females which manage to engorge usually weigh less than normal and lay a reduced number of eggs (Drummond et al., 1981; Wilkins et al., 1981; Lancaster et al., 1982; Jackson and Chesterman, 1989; Soll et al., 1989). IVM also reduces the moulting success rate of nymphs of *A. hebraeum*, *R. evertsi* and *R. appendiculatus* (Centurier and Barth, 1980; Soll et al., 1989).

The mode of action of the AVMs in ticks is still unknown, but the diversity of its effects suggests that it may interfere with hormonal systems. Moulting in ticks is controlled by ecdysteroids (Connat, 1987) and *A. hebraeum* females synthesize large amounts of E and 20-HE following the bloodmeal. These ecdysteroids first appear in the haemolymph and then accumulate in the eggs (Connat et al., 1985). It has been suggested that ecdysteroids may play an important role in vitellogenesis (Diehl et al., 1986), but work from our laboratory suggests that 20-HE is not the vitellogenic hormone (Chapter II). Ecdysteroids also induce degeneration of the salivary

glands after the bloodmeal in adult females (Harris and Kaufman, 1985; Kaufman, 1991).

When injected into the haemocoel of engorged females, AVM B1a and IVM do not prevent salivary gland degeneration in *A. hebraeum* and therefore they probably do not inhibit completely the release of ecdysteroids. However, they virtually abolish egg production at about 100 µg/kg body weight (b.w.; Kaufman et al., 1986). In the latter study, treated females that did not lay eggs nevertheless showed clear signs of vitellogenesis. The ovaries contained the same amount of vitellin and looked very similar to those of normal ovipositing females. The authors thus concluded that the effect of the AVMs was to inhibit oviposition specifically rather than vitellogenesis. They suggested that the retention of developed eggs in the ovary was probably inhibiting further egg production.

In this study, we re-examined the effect of AVMs on ovary development in view of some data which suggested that vitellogenesis was indeed inhibited (see results section). We used the new AVM analogue 4"-epi-methylamino-4"-deoxyavermectin B1 (MK-243) which has greater potency on some insect pests than do earlier AVM products (Dybas et al., 1989).

Materials and methods

1) Ticks

All ticks came from a laboratory colony kept in darkness at 27°C, >95% relative humidity. Adult ticks were fed on rabbits as described by Kaufman and Phillips (1973). Engorged females (>600 mg) were treated less than 24 hours after detachment from the host.

2) Blocking of oviposition by gluing the gonopore

Engorged females were held in the colony incubator for 9 to 10 days after detachment (just prior to the onset of oviposition). Each female was cleaned in tap water and the gonopore was plugged with a droplet of cyanoacrylate glue. The females were returned to the incubator until the day of dissection, 4 to 5 weeks post-engorgement. Their ovaries were then processed as described under *Vitellogenesis and oviposition.*

3) Drugs and injections

MK-243 was a gift from Merck Sharp and Dohme Research Laboratories, Three Bridges, New Jersey. A stock solution (1 mg/ml in 1.2% NaCl) was prepared and stored at -15°C. The stock solution was thawed and diluted to working concentration in 1.2% NaCl just prior to use. All ticks were surface sterilized with ethanol before injection. We injected 1µl/ 100 mg b.w. into the haemocoel with a 25 µl Hamilton syringe. Controls received 1.2% NaCl. The injection was done through the soft articulation between the scutum and the

capitulum. In experiments which monitored oviposition, the injection was done through a cut leg and the wound was sealed with a droplet of cyanoacrylate glue. The latter method prevents damage to Gené's organ, the egg-waxing organ of ticks (Kaufman et al., 1986).

4) Collection of haemolymph and preparation of samples for RIA

Sampling of haemolymph was done as described by Kaufman (1991). Briefly, ticks were glued ventrally to a Petri dish and covered with ice for 15 minutes. Incisions through the integument (about 5 mm long) were made along both sides of the tick. The exuding haemolymph was collected with a volumetric capillary tube and added to 150 µl of methanol in a small microfuge tube. The samples were stored in a freezer at -15 °C until the day of the assay. Just prior to RIA, the samples were vortexed, centrifuged and the supernatant was collected. The pellet was washed twice with 50 µl of fresh methanol. The combined supernatants were then evaporated under vacuum and redissolved in an appropriate volume of borate buffer/BSA (0.05 M borate buffer containing 50 mg BSA/ 100 ml, pH= 8.4).

5) Radioimmunoassay (RIA)

This assay was done as described by Kaufman (1990), using 20-HE as standard. The antibody used in this study was directed against ecdysone-22-succinylthyroglobulin and was diluted to 1/2800, giving a B_0 of 25-30%. Since the antibody cross-reacts with several ecdysteroids, data are reported as pg 20-HE equivalents/µl

haemolymph. Protein A was prepared according to the method of Kessler (1981).

6) *Vitellogenesis and oviposition*

The ovaries and oviducts were exposed and observed under the dissecting microscope in order to evaluate their general stage of development and to measure the length of the biggest oocytes with an ocular micrometer. The ovaries were then gently blotted, weighed, homogenized in 3.4 ml of distilled water and stored at -15°C. On the day of analysis, the samples were thawed, vortexed, centrifuged at 20,000 g for 15 minutes and the supernatant was kept for the spectrophotometric assay. The difference of absorbance between 400 nm (near peak absorbance for the haem moiety of vitellin) and 500 nm (non-specific to haem) reflects the total amount of vitellin in the ovary. This difference of absorbance divided by the weight of the ovary reflects the concentration of vitellin in the tissue sample.

The eggs oviposited were weighed every other day. Total egg weight was expressed as percentage of body weight in order to minimize the variability due to the wide range of engorged weights (from 500 to over 3,000 mg).

7) *Statistics*

Results are reported as mean +/- SEM (n=). N is also recorded on the graphs above the standard error bars. Differences were analyzed using Student's T-test.

Results

1) Effect of blocking oviposition on vitellogenesis

Kaufman et al. (1986) suggested that IVM did not inhibit vitellogenesis directly, but only indirectly via inhibition of oviposition. They proposed that the ovary could expand to a limited extent only, further vitellogenesis being dependent upon oviposition of mature eggs. In the following experiment, ovary size was measured in 5 normally engorged females with plugged gonopores 4 to 5 weeks post-engorgement (see Materials and Methods). Oviposition in this species normally begins 10 to 12 days after detachment. In most specimens with plugged gonopores, the ovary was so distended that it ruptured during dissection, spilling many eggs into the medium. In these cases, we collected as many of these eggs as possible and weighed them with the rest of the ovary. The ovaries accounted for 43 +/- 3 %b.w. (n=5). This figure is in the range of the total egg mass laid by untreated females under normal conditions (see Kaufman et al., 1986). The ovaries of control ticks that were ovipositing normally weighed significantly less (6.6 +/- 0.5% b.w., n=7). Thus, contrary to the suggestion of Kaufman et al. (1986), vitellogenesis appears not to be inhibited by simple blockage of oviposition. These results caused us to reconsider a direct effect of the AVMs on vitellogenesis.

2) Effect of MK-243 on oviposition

Fig. III-1 shows the effects of MK-243 on oviposition latency and egg production. The drug reduced the amount of eggs laid: the egg mass of the controls was 39.4 +/- 6.6 % b.w. (n=8), the egg mass of the ticks treated with 100 µg MK-243/kg was 1.8 +/- 1.4% b.w., 6 out of the 8 ticks producing no eggs at all. MK-243 increased oviposition latency at best only slightly, from 12.3 +/- 0.5 days (n=8) for the controls to 12.8 +/- 0.6 days (n=5) for 50 µg/kg and to 14.0+/- 0.0 days (n=2) for 100 µg/kg; these differences were not statistically significant, the sample size being quite small. The mortality in this experiment was quite high for the treated groups (3 of 8 ticks died after 50 µg MK-243/kg and 6 of 14 after 100 µg MK-243/kg, but no controls died). This experiment shows that 100 µg MK-243/kg virtually abolishes egg production in engorged females so we used this dose to investigate further its mode of action.

3) Effect of MK-243 on ovary development and haemolymph ecdysteroid concentration

MK-243 inhibits or slows down the development of the ovary, as shown in Fig. III-2. Ten days post-engorgement, the ovaries of females treated with 100 µg MK-243/ kg were much smaller than those of controls (0.86 +/- 0.17 % b.w., n=7 versus 5.00 +/- 0.86 % b.w., n=8; Fig. III-2A). They also contained less vitellin, both in absolute amount and in concentration per gram ovary: the difference of absorbance between 400 and 500 nm (reflecting the total amount of vitellin) was 0.136 +/- 0.086 (n=7) for treated ticks versus 1.046

+/- 0.174 (n=8) for controls and the difference of absorbance per gram ovary (reflecting the concentration of vitellin) was 6.43 +/- 2.4 (n=7) for treated ticks versus 11.79 +/- 1.23 (n=8) for controls (Fig. III-2B). The average length of the biggest oocytes (260 +/- 50 µm, n=7) was about half of that of controls (530 +/- 30 µm, n=8; Fig. III-2C). The ovaries of treated females were generally still white or had just a few eggs starting to accumulate vitellin. Only one treated female had larger oocytes and was comparable to the controls, which had larger and browner oocytes covering at least half of the surface of the ovary. Three out of 8 controls were ready to oviposit and eggs were present in their oviducts.

Treatment with MK-243 also reduced haemolymph ecdysteroid concentration to about one-tenth the control value (46 +/- 23 pg/µl versus 491 +/- 75 pg/µl; Fig. III-2D). All the differences were statistically significant (T-test, $p<0.05$). The mortality was quite high in this experiment: 3 out of 11 controls and 13 out of 20 treated ticks died within 10 days.

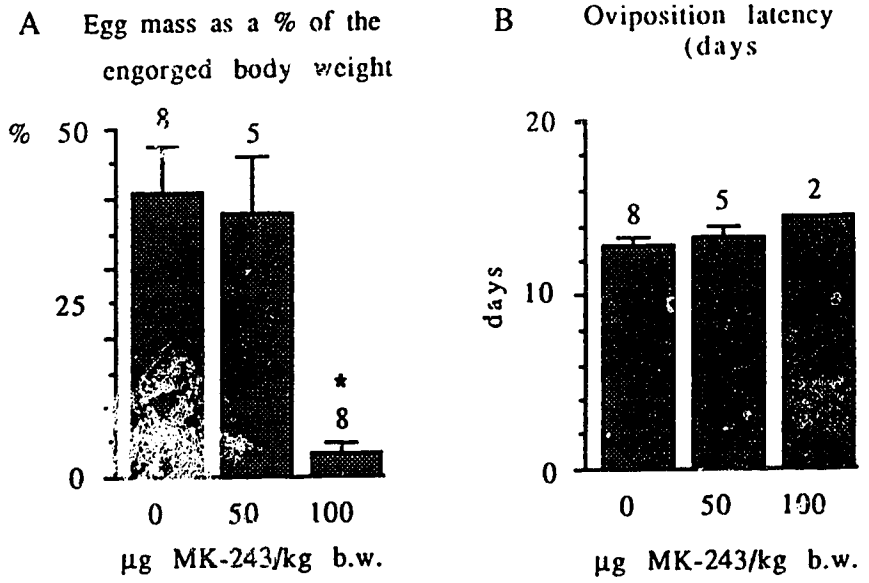

Figure III-1:
Effect of MK-243 on egg production and oviposition latency when injected into fully engorged *Amblyomma hebraeum* females. Ticks were injected with 50 or 100 µg MK-243/kg body weight. Controls were injected with 1.2% NaCl. A) Eggs oviposited as percent body weight,
B) Oviposition latency in days. (*) indicates a significant difference from the control (T-test, $p<0.025$).

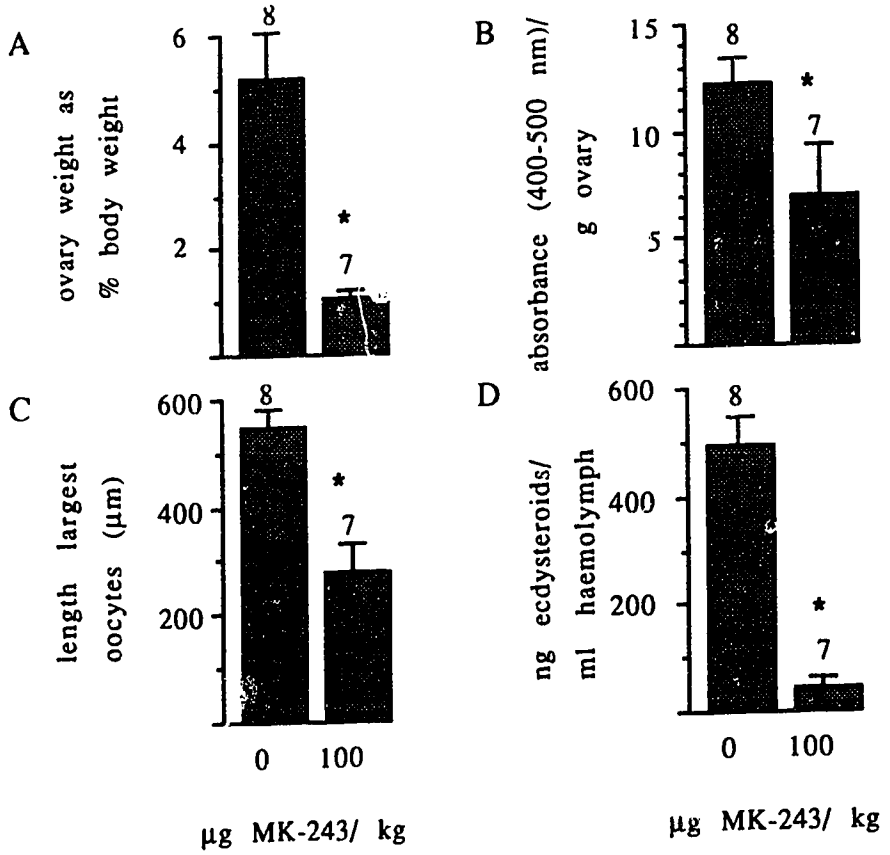

Figure III-2:
Effect of MK-243 on ovary development and haemolymph ecdysteroid concentration 10 days post- engorgement. Freshly engorged females were injected with either 100 µg MK-243/ kg body weight or with 1.2 % NaCl. A) Ovary weight as percent body weight, B) Concentration of vitellin in the ovary , C) Length of the largest oocytes and D) Haemolymph ecdysteroid concentration. (*) indicates a significant difference from the control (T-test, $p<0.05$).

Discussion

The mode of action of AVMs in insects is still unclear, but some authors have suggested that their main effect is to induce paralysis and to inhibit feeding by interfering with GABA neurotransmission (see introduction). The mode of action in ticks is unknown. Ticks feeding on hosts treated with IVM take longer to complete their bloodmeal and usually weigh less than normal when they detach. Mating of females of *Rhipicephalus appendiculatus* is also disrupted (Wright, 1986; Strong and Brown, 1987; Jackson, 1989; Jackson et al., 1989). Since mating triggers the rapid final phase of engorgement in ixodid females and because feeding above a critical weight (about 10 times the unfed weight) is necessary for vitellogenesis to take place (Diehl et al., 1982), it is not possible in the above studies to determine whether AVMs have a direct effect on the maturation of the ovaries or if the reduced mass of eggs is rather a consequence of disruption of mating and feeding. In our experiments, as in the previous ones done in this laboratory (Kaufman et al., 1986), we injected the drugs directly into the haemocoel of mated engorged females and we were able to show that even then, maturation of the ovary was affected: the number of eggs laid was reduced and the oviposition latency was increased. AVMs therefore probably have a direct effect on reproduction in ticks.

In order to assess the general stage of development of the ovaries, we measured three parameters : (a) wet weight of the ovary, (b) its absorbance, which reflects the amount of vitellin it contains,

and (c) length of the largest oocytes. In ticks, the oocytes do not develop synchronously during the gonotrophic cycle; oocytes of all developmental stages are present at any time (Diehl et al., 1982). The less mature ones are found in the longitudinal groove and the biggest and most advanced ones protrude on the other side of the ovary. Oocytes begin to store vitellin and to appear light brown under the dissecting microscope when they reach approximately 200 µm. Typically, the first eggs to reach that stage do so about 4 days after normal engorgement. At this time, the ovary weight, its absorbance and the length of its oocytes start to increase rapidly (Chapter II). These 3 parameters, taken together, define quite well the general state of ovarian development.

There was a rather high mortality for ticks injected with 100 µg MK-243/kg: 43% (treated) versus 0% (controls) died in the experiment monitoring oviposition (Fig. III-1) and 65% (treated) versus 27% (controls) died in the experiment monitoring vitellogenesis (Fig. III-2). The higher mortality in the latter experiment probably reflects a problem in the tick colony or bacterial contamination during the injections at that time because the mortality of controls was also quite high. The impairment of the reproductive cycle that we observed may therefore reflect a general morbidity of the surviving ticks. However, all the ticks that survived the pre-oviposition period were still alive more than 5 weeks after detachment and 2 of them were still alive after 70 days.

By blocking the gonopore of engorged females with cyanoacrylate glue, we showed that the retention of mature eggs in

the ovary did not inhibit vitellogenesis. Instead, mature eggs accumulated in the ovaries, which accounted for about 43% of initial body weight when dissected 4 to 5 weeks after detachment. This was in the range of the total egg mass produced by untreated ticks after a similar duration. This result contradicts the interpretation offered by Kaufman et al. (1986) to explain reduced fecundity of IVM-treated females (see Introduction) : since the retention of mature eggs in the ovary does not appear to prevent further egg production, it is more likely that the AVMs inhibit vitellogenesis itself. The precise mode of action of AVMs remains to be determined: it could inhibit (1) synthesis of vitellin, (2) uptake of vitellin by oocytes, (3) synthesis of the vitellogenic hormone or (4) afferent neural pathways to the endocrine organ which secretes the vitellogenic hormone (unknown in ticks). Glancey et al. (1982) reported that AVM B_1 causes irreversible cell and tissue damage to the ovaries of the red imported fire ant queens *Solenopsis invicta*, which result in complete sterility or in a reduction of the number and size of the eggs. It would be most interesting to do a histopathological study to see if there is also direct damage done to the ovaries of *A. hebraeum* treated with MK-243.

Although we showed that the effects of MK-243 cannot be explained simply by a blockage of oviposition, AVMs may inhibit oviposition as well. There is some evidence pointing to the role of GABA as an inhibitory neurotransmitter in the neuromuscular junction of the dorso-ventral muscles and in the central nervous system of *A. hebraeum* (Gration et al., 1986). It is therefore

conceivable that AVMs paralyse muscles responsible for oviposition. Some circumstantial evidence supports this hypothesis : when we dissected 2 females treated with 100 µg MK-243/ kg 70 days after detachment from the host, their ovaries accounted for 15% and 40% b.w. respectively. This is much higher than the ovary weight for normal ovipositing females (approximately 5% b.w.); despite the accumulation of eggs in their ovaries, these females still weren't ovipositing.

MK-243 reduced, in a dose-dependent manner, the amount of eggs laid and its efficacy was comparable to that of IVM (Kaufman et al., 1986). However, its effect on oviposition latency was minimal and the slight increase we observed is probably not statistically significant. AVM B1a and IVM both increased oviposition latency by several days in the study of Kaufman et al. (1986).

An interesting finding in this study was that MK-243 slowed down ovarian development even before the beginning of oviposition, which confirms our earlier suggestion that AVMs act on vitellogenesis itself: ten days after detachment from the host, the ovaries of treated ticks were much smaller, contained much less vitellin and their most mature oocytes were about half the size of controls (Fig. III-2). Actually, the ovaries of these treated ticks after 10 days were comparable to those of untreated ticks 4 to 5 days after detachment (see Chapter II).

Ticks treated with MK-243 also had a haemolymph ecdysteroid concentration about one-tenth that of controls, a concentration which is normally found in engorged females only 2 to 3 days after

detachment (Connat et al., 1985; Kaufman, 1991). This finding is of interest because 20-HE has been proposed as the vitellogenic hormone in ticks (Diehl et al., 1986). If the latter were true, AVMs could inhibit vitellogenesis by interfering with the hormonal system, either directly or through a hypothetical GABA system in the central nervous system. However, there is no published evidence that ecdysteroids trigger vitellogenesis in ticks, and the data in Chapter II likewise fails to demonstrate a specific role for ecdysteroids.

20-HE also triggers salivary gland degeneration in engorged ixodid females. In our experiments, despite the low concentration of this hormone in females treated with MK-243 (46 +/- 23 pg/µl), their salivary glands had degenerated. Kaufman (1991) has shown that salivary glands of ticks degenerate within 7 days in the presence of less than 100 pg 20-HE/µl haemolymph. Although haemolymph titers of treated ticks in this study were only about 50 pg/µl, this may have been sufficient to cause significant degeneration within 10 days. (The threshold concentration for inducing salivary gland degeneration after 4 days in organ culture can be as low as 30 pg/µl; Harris and Kaufman, 1985). Kaufman et al. (1986) also reported that treatment with AVM or IVM does not prevent salivary gland degeneration, even at doses sufficient to block oviposition.

In conclusion, our data show that MK-243 inhibits ovary maturation and vitellogenesis and lowers haemolymph ecdysteroid concentration of engorged *A. hebraeum* females. These effects are

not due to a disruption in feeding, mating nor primarily to the blockage of oviposition.

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CHAPTER IV

GENERAL DISCUSSION AND CONCLUSIONS

The information available on the hormonal control of vitellogenesis in ixodid ticks was rather scarce at the beginning of this study, and can be summarized as follows. E and 20-HE circulate in the haemolymph during vitellogenesis in *Amblyomma hebraeum* and their titers increase from engorgement to the onset of oviposition. These ecdysteroids then accumulate in the eggs (Connat et al., 1985). They do not appear in females that are removed from the host before they reach the critical weight and these females do not lay any eggs (Kaufman, 1991). JHs have never been positively identified in ticks, but several experiments suggest their existence in ixodid ticks. A JH-like compound is present in the haemolymph of vitellogenic *Boophilus microplus* females, but its chemical structure is different from any known insect JH or MF (Connat, 1987). In addition, precocenes reduce fertility in at least 2 ixodid species (Leahy and Booth, 1980; Connat, 1988). Although this evidence suggests that ecdysteroids and/or a JH-like compound are present during the gonotrophic cycle of ixodid ticks, it fails to assign a specific function to these hormones. Possible roles include the control of vitellogenin synthesis, of its uptake by the ovary, of oviposition, of lipid synthesis by Gené's organ for waterproofing of the eggs, of early embryogenesis, and perhaps others (Diehl et al., 1986). The purpose of the experiments in this study was to test directly whether an

ecdysteroid or a JH is the hormone that triggers ovary growth and vitellogenesis in engorged *Amblyomma hebraeum*.

By transplanting ovaries of small partially fed females into the haemocoel of engorged females and showing subsequent development, I was able to demonstrate that vitellogenesis is triggered by a factor circulating in the haemolymph of engorged females. Ovaries are already able to respond to this factor when ticks are still small. 20-HE and JH analogues both failed to mimic the effect of this factor.

JH III and the two JH analogues MF and JHB3 were unable to stimulate ovary growth and vitellogenesis in both partially fed and engorged females, and P II did not inhibit vitellogenesis in engorged females. These experiments failed to suggest a role for JH in control of vitellogenesis, but the involvement of JH-like compounds still cannot be ruled out for the following reasons: first, the concentration of JH or P II in the haemolymph after the treatments was not measured and may not have been adequate to initiate a response. Secondly, as suggested already by Connat (1987), the chemical structure of tick JH, if it exists, may be quite different from any known JH; our JH analogues may have been ineffective for this reason.

20-HE was unable to stimulate ovary growth or vitellogenesis when infused into the haemocoel of small partially fed females, even though the concentration used was high enough to induce complete salivary gland degeneration. The hormone seemed to induce proliferation of the fat body and could therefore play an important

role in vitellogenesis by controlling fat body maturation and perhaps also vitellogenin synthesis, but this needs further investigation. 20-HE was also unable to accelerate or amplify vitellogenesis in engorged females, even though its concentration in the haemolymph was increased considerably after the infusion. 20-HE is therefore unlikely to be the hormone controlling vitellogenesis in ixodid ticks.

The avermectin MK-243 slowed down vitellogenesis and reduced the haemolymph ecdysteroid concentration to one-tenth the control values. The evidence available from this thesis suggests that the two reductions may be independent of each other. Also, we do not know what specific step of vitellogenesis MK-243 inhibited: it could be, for example, the synthesis of the vitellogenic hormone, the synthesis of vitellogenin, or its uptake by the oocytes.

Although I was unable to demonstrate that 20-HE or JH control vitellogenesis on their own, I cannot exclude the possibility that they may control part of the process, in association with other compounds. The methods I used were designed to detect only the uptake of vitellin by the ovary, but not trace amounts of vitellogenin in the haemolymph. I therefore suggest a series of experiments that could bring valuable information on the respective roles of 20-HE and JH in vitellogenesis and on the mode of action of MK-243.

1) An antibody directed against *Amblyomma hebraeum* vitellogenin should be raised in order to measure the concentration of vitellogenin in the haemolymph, the fat body and the midgut cells of small partially fed females after treatment with 20-HE or JH.

Stimulation of vitellogenin synthesis might then be detected even if it is not absorbed by the developing oocytes.

2) The effect of 20-HE on the fat body mentioned in chapter II (page 39) must be followed up: ultrastructural changes induced in the fat body trophocytes of small females after infusion with 20-HE should be examined and compared with those normally occuring after the bloodmeal.

3) In order to test if JH or 20-HE stimulates the uptake of vitellin in the oocytes, the ovaries of partially fed or engorged females should be cultured in vitro in the presence of a source of vitellin, with and without 20-HE and JH III. Uptake of vitellin could be assessed by measuring the absorbance of the homogenized ovaries, by immunostaining of electron microscopy sections with the antibody raised against vitellogenin or by labelling vitellin with a radioactive isotope. Ultrastructural examination of the ovaries would also reveal any stimulation of the formation of micropinocytotic vesicles for vitellogenin uptake.

4) The nature of the vitellogenic hormone should also be investigated directly. Although injection of fresh haemolymph from engorged females into small ticks failed to stimulate vitellogenesis, similar experiments should be attempted again. Multiple injections of fresh haemolymph (perhaps treated with anti-oxidants) or treatment of small females with haemolymph extracts might give better results.

Stimulation of vitellogenesis by haemolymph extracts could also be measured with the antibody raised against vitellogenin. If one of these treatments proves effective, the haemolymph should be fractionated further by HPLC. The vitellogenic hormone might then be isolated and identified.

5) Finally, in order to investigate further the mode of action of MK-243, one could try to reverse its anti-vitellogenic effect by infusing treated ticks with 20-HE. A positive result would show that ecdysteroids are somehow involved in regulation of vitellogenesis. The concentration of vitellogenin in the haemolymph after treatment with MK-243 could also be measured to see if the product acts at this level. Also, the ovaries of treated females should be examined by electron microscopy in order to see if MK-243 induces cytopathological changes that could account for the slower development of this organ in treated females.

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