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Influence of Copulation on Vitellogenesis and Egg-laying in *Ornithodoros moubata* Murray (Ixodoidea : Argasidae)[1]

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I. Introduction

The influence exerted by copulation on vitellogenesis and egg-laying in ticks has not been extensively studied (Galun and Warburg, 1967; Aeschlimann, 1968; Pappas and Oliver, 1971, 1972; Leahy and Galun, 1972; Aeschlimann and Grandjean, 1973; Eisen *et al.*, 1973; Khalil and Shanbaky, 1975; Shanbaky and Khalil, 1975; also, see review by Oliver, 1974). It is evident from these studies, dealing principally with the Argasidae, that responses to different stimuli vary somewhat according to the species under consideration. Thus, in *Ornithodoros tholozani,* a catecholaminergic substance in the seminal fluid induces the onset of vitellogenesis but does not trigger egg-laying (Galun and Warburg, 1967). *O. tholozani* does not respond to a mechanical stimulus. By contrast, Leahy and Galun (1972) showed that in *Argas persicus,* both mechanical (placing of beads in the vagina) and chemical (injection of a complex of male glandular tissue) stimuli can induce vitellogenesis. Yolk deposition is never complete, however, and egg-laying does not occur. The presence of spermiophores in the female genital tract is indispensable for oviposition in these two species.

The story is different with *O. moubata.* On injection of spermiophores (either inact or as homogenate) directly into the body cavity, virgin females complete vitellogenesis and the eggs are laid (Aeschlimann, 1968). This

1 We dedicate this paper to the memory of Prof. Jean-G. Baer who honoured us by reading the manuscript before his death.

suggests that in the spermiophores of this species there exists an activating substance(s) for vitellogenesis and oviposition[2].

On the other hand, it would appear that the events which lead to oviposition may be mediated in ticks, as in insects, by the endocrine system (Aeschlimann and Grandjean, 1973; Eisen *et al.*, 1973), but this has not yet been conclusively demonstrated. In the present study we have attempted to determine the nature and origin of the stimulus triggering vitellogenesis and egg-laying in *O. moubata*. The results obtained todate are still incomplete, but they enable us to present some hypotheses which are currently being tested in our laboratory (Ducommun, in preparation).

II. Materials and Methods

A. Tick Rearing

O. moubata used in this study originated in Tanzania and were reared in Neuchatel according to the method of Geigy and Herbig (1955) at 26–28°C and 80–90% RH. To ensure that we obtain virgin adults, after feeding the 4th nymphal stage, we segregated the animals in glass tubes stoppered with cotton-wool plugs. The sexes were then fed separately on guinea-pigs. Ten to 20 days following engorgement and copulation (the preoviposition period) egg-laying occurs.

B. Application of Stimuli

1. *The Normal Condition*

Vitellogenesis and egg-laying are normally triggered by feeding and copulation. Copulation is characterized by the introduction in the female genital tract of a spermatophore containing male gametes or spermiophores.

2. *Delayed Copulation*

In the laboratory, copulation can be delayed for several weeks or months from the time of engorgement. Each virgin female is then enclosed in a tube together with two or three virgin males. Whenever possible, mating was confirmed by observation. One cycle of egg-laying can take place following this "delayed copulation" (Aeschlimann, 1968).

3. *Artificial Induction*

Artificial induction of vitellogenesis and egg-laying can be achieved by the injection of spermiophore extracts taken either from the seminal vesicles of the male or from the uteri of mated females where the spermiophores undergo maturation. The spermiophores obtained by dissection were subsequently exposed to one of the following treatments :

(a) *Homogenization.* The spermiophores were homogenized in physiological saline (0·8% NaCl) with a glass micro-homogenizer kept on ice. The homogenate was injected into virgin females.

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2 We emphasize that the results of these injections permitted us to confirm not only the existence of limited parthenogenesis in *O. moubata*, but also to demonstrate the possibility of autogenesis in the Ixodoidea (Oliver, 1971 and Feldman-Muhsam and Havivi, 1973 for reviews).

(b) *Incubation.* The spermiophores were suspended and washed 2 or 3 times by low speed centrifugation in physiological saline in order to rid them of male or female accessory gland secretions. The resultant suspension could then be injected into virgin females. Spermiophores are not damaged by this treatment. After washing, it is possible to keep the spermiophores alive in 0·8% saline solution (28° C) for at least 20–50 hr. After incubation and centrifugation, both spermiophores and supernatant can also be injected into virgin females. Examination of the sediment under the microscope adjusted for dark field revealed that some spermiophores are still viable.

(c) *Injections.* Injections were made via the intersegmental membrane between the coxa and trochanter of legs III and IV by means of a fine glass capillary attached to a syringe. *O. moubata* will tolerate in its haemolymph at least 10 µl of fluid. In these experiments we injected 5 µl. The use of crude extracts did not allow strictly quantitative work. In general, each female received the equivalent of 1/10 to ½ a spermatophore, or the equivalent of 1/10 to ½ of the male genital complex. The injection of larger quantities was toxic.

C. Detection of The Responses to a Stimulus

In these experiments our criterion for a positive response to a stimulus was subsequent oviposition or vitellogenesis. Females which did not lay eggs 30 days after receiving a natural or artificial stimulus were dissected in order to inspect the state of their ovaries. White oocytes (0·15–0·20 mm diameter) are free of yolk. Slightly larger light brown oocytes have begun vitellogenesis. The mature egg is dark brown and measures 0·8–1·0 mm in diameter (Fig. 1A). At this stage it may still be attached to the ovarian wall, or may be found in the lumen of the ovary, oviduct or uterus. The brown colour is derived from haemoglobin metabolites incorporated into the egg by micropinocytosis (Wigglesworth, 1965; Aeschlimann and Hecker, 1967, 1969; Diehl, 1970; Jenni, 1971). Thus two parameters (size and colour of the eggs) permit diagnosis of the triggering of vitellogenesis by a stimulus.

III. Results

The experiments are summarized in Table I. Some of them extend our observations of 1968; others are original.

A. Controls

1. *Females Fertilized after Feeding*

Females engorge within 20 to 60 min on guinea-pigs and copulate on the same day. All females lay viable eggs after a normal preoviposition delay of 10–20 days. Vitellogenesis takes place during this period. The number of eggs laid varies from 80 to 150 per female. *O. moubata* shows much variability among the individuals, with regard to the number of eggs laid, and the preoviposition period. Nevertheless, feeding and copulation are the prerequisites for egg-laying after a normal preoviposition delay.

2. *Virgin Females after Feeding*

Virgin females also engorge, but do not normally lay eggs. However, feeding does induce vitellogenesis which may proceed to a considerably advanced stage before the yolk is resorbed (see below). In 10–20% of the

gorged virgins, a few eggs reach maturity and ovulation can take place. On dissection, one sees these eggs in the lumen of the genital tract. A small number of these eggs (less than 30) are laid, but after an unusually long preoviposition period (30–50 days). These eggs are not viable, although a few (5–15%) can develop by parthenogenesis. In virgin females which do not lay after a meal, resorption of the yolk is seen after the 25th day; degenerating eggs are present within the ovarian tissue or in the lumen of the genital duct. The eggs have an irregular form; the yolk which first appears granular seems to liquify subsequently. In the end, all that remains visible is a minute, whitish residue of a membranous nature which can sometimes be seen several weeks after feeding, particularly in the genital ducts (Fig. 1B). A thorough study of this degeneration phenomenon in *A. persicus* has been published by Leahy and Galun (1972). Our observations agree with those of Leahy and Galun (1972). The biological mechanisms that control oosorption are unknown.

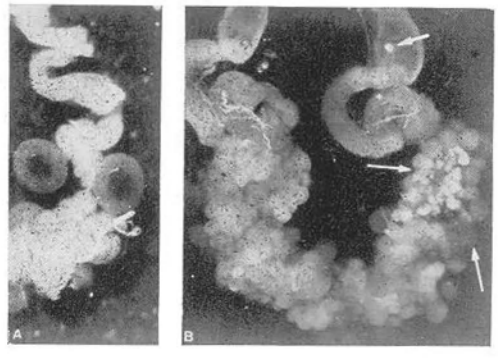

Fig. 1. A. Ovary 10 days after copulation and nutrition, Note the two well developed eggs, and eggs in various stages of vitellogenesis. B. Ovary of a virgin female, 30 days after nutrition. Many eggs are undergoing resorption (thin arrows). The white residue of a completely resorbed egg is seen in the ampulla of the oviduct (thick arrow).

3. *Virgin Females 90 Days after Feeding*

Dissections of virgin females which had not laid by 90 days after feeding, revealed that their ovaries were in a juvenile state, as in virgin females which had never fed. The oocytes are small, whitish and without a trace of yolk. Resorption of those eggs which had undergone vitellogenesis is by this time complete. One can detect the residues mentioned above. Ninety days after feeding, only one out of 52 females still possessed visible yolk in a few oocytes in the process of degeneration. In conclusion, this series of preliminary experiments attests that the taking of a blood meal leaves no

TABLE I

Influence of Copulation and Injections of various Materials on Vitellogenesis and Egg-laying in *O. moubata*

No. of the experiment	No. of ♀♀ used	Condition of ♀♀ at the beginning of the experiment	Treatment of ♀♀ (copulation or injection)	No. of ♀♀ that laid (a)	No. undergoing vitellogenesis (b)	Total responding to stimulus (a & b)	No. not responding to stimulus	Preoviposition delay (days)
1	25	Gorged followed by copulation the same day		25		25	0	10–20
2	28	Engorged virgins		4 (but < 30 eggs each)	not observed			33–48
3	52	Virgins engorged 3 months previously		0	1 (oocytes degenerating) 51 (white oocytes without yolk)			
4	14	,,	normal copulation	14		14	0	6–14
5	10	,,	injection of 0-8% NaCl	0	0	0	10	
6	33	,,	injection of seminal vesicle homogenate	31	2 (strong vitellogenesis)	33	0	11–19
7	34	,,	injection of mature spermiophores	32	2 (strong vitellogenesis)	34	0	10–16

TABLE I (continued)

No. of the experiment	No. of ♀♀ used	Condition of ♀♀ at the beginning of the experiment	Treatment of ♀♀ (copulation or injection)	No. of ♀♀ that laid (a)	No. undergoing vitellogenesis (b)	Total responding to stimulus (a & b)	No. not responding to stimulus	Preoviposition delay (days)
8	10	,,	injection of 20 hr incubated spermiophore	10		10	0	12–20
9	55	,,	injection of 20 hr incubation medium	55		55	0	9–20

TABLE II

Influence of Temperature on the Activity of the "Active Substance(s)"

No. of ♀♀ used (gorged 3 months previously)	Injection of incubation medium subjected to various temperatures	No. of ♀♀ that laid	No. of ♀♀ undergoing vitellogenesis (but not laying)	No. responding to stimulus	No. not responding to stimulus	Preoviposition delay (days)
12	20 hr at 0° C	10	1	11	1	8–20
15	20 hr at 30° C	10	3	13	2	10–20
10	20 hr at 45° C	2	2	4	6	7–10
10	20 hr at 60° C	0	0	0	10	—
15	10 min at 100° C	0	0	0	15	—

traces of yolk in virgin females 90 days after feeding. However, as the following experiment demonstrates, such females still possess sufficient nutrient reserves necessary to permit vitellogenesis and a normal laying cycle.

4. *Females Fertilized 90 Days after a Meal*

Ninety days after feeding when the oocytes of virgin females are again in a juvenile state, copulation triggers egg-laying after a normal, or sometimes even after a surprisingly short preoviposition delay. The number of eggs laid is normal. In this case, we assume that "delayed copulation" mobilizes nutrient reserves stored in the body of the female, and triggers vitellogenesis. Delayed copulation possibly exerts its stimulating action either mechanically (for example, by the introduction of the hypostome in the vagina), or else by chemical means. Virgin females, fed once 3 months beforehand, but having never laid, posses oocytes without yolk. Endowed with sufficient nutrient reserves, these females can respond to diverse stimuli (a new feeding excluded). If egg-laying has not begun after a delay of 20 days (which we consider is a normal period for preoviposition), dissection permits the disclosure as to the existence or otherwise of vitellogenesis.

B. Experiments Involving Artificial Stimulation

1. *Injection of Physiological Saline*

The injection directly into the haemocoele of virgin (v) and gorged (g) females 3 months previously (=♀♀ v. g.) of 5μl 0.8% NaCl results in neither laying nor vitellogenesis.

2. *Injection of Homogenates of Seminal Vesicles*

An homogenate of seminal vesicles distended with immature spermiophores was injected into 33 ♀♀ v. g.. Of these, 31 laid after a normal preoviposition period, 2 did not lay, but revealed on dissection 20 days following the injection, ovaries with numerous oocytes filled with yolk. All of these ♀♀ v. g. thus reacted to a stimulus of "homogenized immature spermiophores". It is pertinent to recall at this point that the remainder of the male genital system (testes and accessory glands) seems not to have the same effect on vitellogenesis (Aeschlimann, 1968).

3. *Injection of Spermiophores*

An injection of mature spermiophores taken from the uteri of fertilized females leads to a comparable result, namely, 32 out of 34 ♀♀ v. g. laid eggs after a normal preoviposition delay. The two females which did not lay revealed on dissection that vitellogenesis had proceeded to an advanced stage. Thus all ♀♀ v. g. react to a stimulus of "mature spermiophores".

4. *Injection of Incubated Spermiophores*

The injection of mature spermiophores, also taken from the uterus of fertilized females, but then incubated for 20 hr and finally washed twice in 0.8% NaCl solution provoked laying in all 10 ♀♀ v. g.. This reaction to the stimulus "20 hr incubated spermiophores" occurs after a normal delay.

5. *Injection of Incubation Medium*

The injection into 55 ♀♀ v. g. of medium in which mature, washed spermiophores had rested for 20 hr caused laying to proceed, in all cases after a normal preoviposition period. That spermiophores exert their influence by chemical means is thus established beyond reasonable doubt.

We have attempted two preliminary assays to analyze the liquid medium with a view to characterize the "active substance": (A) As the temperature is increased, the activity of incubation medium is lost (Table II); (B) One assay showed that the passage of incubation medium through a column of Sephadex G 100 permits the recovery of activity in the void volume. Testing each fraction of the column on ♀♀ v. g., we found a peak of activity corresponding to substances excluded by Sephadex G 100. Injection of this phase triggers normal laying or at least advanced vitellogenesis in experimental females. As it is excluded by Sephadex G 100, the active substance may be associated with a molecule of approx. 150,000 daltons. Since it is inactivated by high temperature the active substance is likely to be a protein or a protein complex.

C. Specificity of The Stimulus, "Mature Spermiophores"

Table III demonstrates that the stimulus which triggers vitellogenesis and laying in O. *moubata* is not species specific. Injection of spermiophores of other tick species in the haemocoele of ♀♀ v. g. *O. moubata* also induces laying. Although the spermiophores of *O. tholozani* exerts a more modest influence on the maturation of *O. moubata* eggs, the effect of *O. tartakowsky* spermiophores is striking : by 12 days after treament, all the *O. moubata* had laid.

TABLE III

Influence of Spermiophores of *O. tholozani* and *O. tartakowsky* on Vitellogenesis and Egg-laying in *O. moubata*

Injection into *O. moubata* of mature spermiophores from:	No. of *O.moubata* used	No. of ♀♀ that laid	Preoviposition delay (days)
O. moubata (control)	17	17	10–15
O. tholozani	18	10	9–18
O. tartakowsky	16	16	7–12

IV. Discussion and Conclusions

In *O. moubata* both feeding and copulation are necessary for the laying of viable eggs. This takes place 10 to 20 days after the blood meal (period of oviposition) and the number of eggs laid varies from 80 to 150. The feeding of virgin females always causes vitellogenesis, but in the majority of cases laying does not occur, and by 3 months after the meal the yolk has been resorbed within the female genital tract. A small minority of gorged virgin females which do lay eggs, do so only after an unusually long preoviposition period (30–50 days) and the number of extruded eggs is low, usually below 30. Copulation of gorged virgin females 3 months after feeding also results in normal vitellogenesis and laying after the usual preoviposition delay. We assume that copulation unleashes the mobilization of nutrient reserves stored up by virgin females from the previous blood meal, thus ensuring vitellogenesis, and almost always, egg-laying. We suggest that endocrines probably play a role in this relay of events.

By means of injection directly into the body cavity of virgin females fed 3 months beforehand, of living or homogenized spermiophores, or of incubation medium in which mature spermiophores had been suspended, it was possible to obtain vitellogenesis and laying as in the case of delayed copulation. In these experiments, the preoviposition delay and the number of eggs laid are normal. Obviously, we are dealing with a chemical stimulus. It could be hypothesized that this stimulus is in the form of a substance perhaps secreted by the spermiophore, or at the very least, that it is contained within the spermiophore. A few tests show that this substance is thermolabile, and that it is a protein or a protein complex of a high molecular weight. The active substance is not species specific since extracts from two other *Ornithodoros* spermiophores initiate vitellogenesis and laying in *O. moubata*.

It is interesting to note that the phenomena observed in the argasids (*O. moubata, O. tholozani, A. persicus*) are different from those met with in the ixodids. In the argasids, copulation is a prerequisite for vitellogenesis and egg-laying; in the ixodids, copulation is necessary before the females will feed to completion (Aeschlimann and Grandjean, 1973). In effect, copulation takes place on the host during the first phase of engorgement and it is that act which triggers the final phase of engorgement in females. By experimentally inhibiting copulation of attached females one, by necessity, also inhibits final engorgement. It seemed, in the past, that ticks of the genus *Ixodes* were unique in that females are able to gorge completely without the presence of males on the host. However, recent experiments have shown that in *I. ricinus* copulation occurs during the free phase before females find their host (Graf, 1974). In fact, all females captured in the wild, and which gorge to completion in the laboratory have been fertilized

beforehand. Meeting of the sexes in the vegetation is facilitated by a pheromone secreted by the females (Graf, 1974). Thus in the Ixodidae, copulation intervenes before feeding or facilitates the final phase of engorgement of the female, and the nutriment thus obtained is used for vitellogenesis. With the Argasidae, copulation is necessary only after feeding to ensure vitellogenesis and egg-laying; it thus does not play a role in the nutrition of the female.

One can compare these phenomena with those known for insects. In several species of insects (see reviews by Davey, 1965 and Engelmann, 1970) copulation is the stimulus that triggers the hormones responsible for vitellogenesis. On the other hand, nutrition can act equally on the endocrine system which in turn releases the gonadotropic hormones. The presence of an endocrine relay in the argasids is suggested since it is possible to induce vitellogenesis and laying by the transplantation of "active" brains or by injection of homogenized brains, directly into the haemocoele of virgin female *O. moubata* (Aeschlimann, 1968; Shanbaky and Khalil, 1975). Several groups of neurosecretory cells have, moreover, been demonstrated in the central nervous system of ticks by many authors (Ioffe, 1963, 1964, 1965; Dhanda, 1967; Eichenberger, 1970; Binnington and Tatchell, 1973; Eisen *et al.*, 1973; Roshdy *et al.*, 1973; Obenchain, 1974a, b; Obenchain and Oliver, 1975). One can, therefore, visualize the two following chains of action, one valid for the ixodids and the other for the argasids (Fig. 2). Obviously these are only hypotheses, based on recent observations, of which the elements representing cause and effect have not all yet been proven.

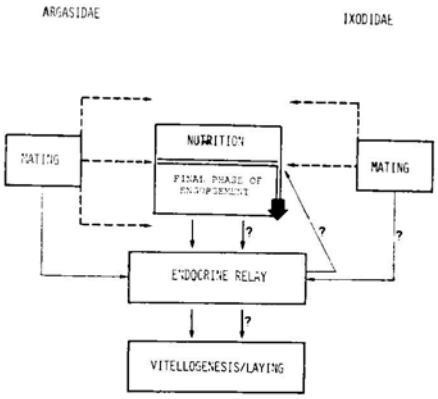

Fig. 2. Reproductive cycle in ticks. Argasidae: In the laboratory, mating (— — — →) may occur before, during or after the blood meal. Ixodidae: In the laboratory as in the wild, mating may occur before the blood meal (*I. ricinus*), or on the host. It triggers the final phase of engorgement (thick arrow). Arrows marked with (?) mean that the relation is not yet proven.

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