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Original article

**Toluidine blue staining as a rapid measure
for initiation of oocyte growth and fertility
in *Varroa jacobsoni* Oud.**

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Abstract – A whole mount method was used to determine the moment of activation of oocyte growth in *Varroa jacobsoni* females. Ovaries of the mites were dissected and stained with toluidine blue. The coloration of the terminal oocyte indicates the uptake of euplasmatic and/or yolk material and, therefore, the initiation of the reproductive phase. In phoretic mites from adult bees, no staining of the ovary could be detected. *V. jacobsoni* females artificially introduced into freshly capped brood cells and removed for dissection 6 h later already showed clear blue staining of the terminal oocyte. The ovaries of *V. jacobsoni* females introduced 14 h after capping of the brood cell, however, remained uncolored after incubation in toluidine blue. We conclude that, in phoretic mites, oogenesis is arrested at a previtellogenic phase. Immediately after invasion of the brood cell, reproduction is activated by an as yet unknown host factor. This factor is present in freshly capped brood cells but not in brood cells 14 h after capping. Our new method offers new possibilities for the exact determination of oocyte growth and, therefore, for the study of *V. jacobsoni* fertility in different host and parasite populations.

***Varroa jacobsoni* / host-parasite relationship / vitellogenesis / oogenesis / fertility / reproduction**

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1. INTRODUCTION

The fertility of *Varroa jacobsoni* mites (Acari, Varroidae) reproducing in worker brood cells has been shown to affect most strongly the population dynamics of the parasite in honey bee colonies [11]. In the original host species, *Apis cerana* Fabr., female mites do not reproduce in worker brood but they have high fertility in drone brood, which they prefer over worker brood [2, 6, 14, 21]. In the secondary host *Apis mellifera* L., reduced fertility of the mites is correlated with increased tolerance to varroosis, as seen in the Africanized honey bees of Brazil [7, 15, 17].

The signals triggering the activation of oocyte maturation of the parasite after invasion of the brood cell are as yet unknown. In phoretic mites, oogenesis is arrested at an early stage of egg cell development. Oocyte growth in reproductive *V. jacobsoni* females depends on the consumption of haemolymph from freshly sealed larvae [10, 25]. These haemolymph meals were assumed to initiate lyrate organ function and, later on, to lead to vitellogenesis. Artificial introduction of mites into sealed brood cells containing a prepupa, however, failed to induce mite reproduction [4, 20]. The supposed stimulation by host derived hormone [12] or other trophogenous factors [20] has not been confirmed [19, 21]. Recent studies indicate a stage-specific semi-chemical stimulation of *V. jacobsoni* reproduction [26]: the polar fraction of host larval cuticle induced egg maturation, though only if extracted from larvae of the fifth instar.

In order to evaluate the moment of effective stimulation and subsequent growth of *V. jacobsoni*'s terminal oocyte, histological examination with time-consuming serial sections were required [22, 23]. Here we present a method for rapid toluidine blue staining of ovary whole mount dissections. By means of this technique, we provide a sensitive method for monitoring the initiation of oocyte growth in *V. jacobsoni*.

2. MATERIALS AND METHODS

2.1. Artificial introduction of *V. jacobsoni* females

Studies were performed at the apiary of the University of Hohenheim with *Apis mellifera carnica*. Phoretic mites were taken from living nurse bees on combs with open brood. A brood comb of another, lightly infested honey bee colony with recently sealed worker brood cells was used for artificial infestation. A small gap in the cell sealing was opened with an insect needle and carefully closed again after introduction of one *V. jacobsoni* female. All mites were introduced within 30 minutes after removal from the adult bees. They were separated into two groups.

2.1.1. "Early introduction"

V. jacobsoni females were introduced into recently sealed brood cells (0–2 hours). Using this method, 80 to 90 percent of *V. jacobsoni* females laid eggs, similar to the percentage in naturally invaded cells at our apiary [16, 20].

2.1.2. "Delayed introduction"

V. jacobsoni females were introduced into brood cells that had been sealed for 14–16 hours. Less than 10% of these mites are able to lay eggs [Rosenkranz and Stürmer, unpublished data]. Therefore, these mites represent a group of "artificially infertile" *V. jacobsoni* females.

In our test colony, naturally infested worker brood cells were checked 7–9 days post capping for *V. jacobsoni* reproduction to ensure that, in the *V. jacobsoni* population we used for our experiments, rates of mite reproduction were normal.

2.2. Removal of mites and record of body weight

After artificial introduction, *V. jacobsoni* females were removed at 6-hour intervals

(6, 12, 18 and 24 hours) from brood cells and immediately weighed on a Mettler fine balance (0.1 mg accuracy) in groups of 5 mites to record average body weight. Phoretic mites were weighed in the same way. For each data point, at least 20 groups of mites were weighed.

2.3. Dissection, whole mount staining, and microscopic analysis of *V. jacobsoni* ovaries

V. jacobsoni females sampled from adult bees or from brood cells were opened by removing the ventral shield. The ovary together with spermatheca and lyrate organ were carefully transferred into PBS buffer (phosphate buffered saline, pH 7.2–7.4). After 30 minutes fixation in formaline (4%), the reproductive tracts were washed three times with buffer and incubated for 30 minutes in toluidine blue (0.005%). After further washing in PBS (three times 15 minutes) the coloring of the oocytes in the ovary was analyzed with a Zeiss photomicroscope (400 \times). In this study, we distinguished between colored and non-colored oocytes to determine whether there had been incorporation of euplasmatic material and/or yolk proteins into the oocyte; this would indicate an activation of the oocyte.

3. RESULTS

3.1. Reproduction in naturally infested brood cells

From 15 worker brood cells of our test colony naturally infested with a single *V. jacobsoni* female, 14 mites laid eggs, indicating that the respective *V. jacobsoni* population had a normal reproductive capacity.

3.2. Change in *V. jacobsoni* weight

Phoretic mites weighed 325 μg ($\pm 26 \mu\text{g}$). Body weight increased to 411 μg ($\pm 30 \mu\text{g}$, “early introduction”) and 407 μg ($\pm 27 \mu\text{g}$, “delayed introduction”) 24 hours after introduction to a cell. During this first day of the reproductive cycle, there was no significant difference ($p > 0.05$, Friedmann-Test) in weight change between the two groups of *V. jacobsoni* mites (Fig. 1).

3.3. Oocyte growth

We analyzed ovaries of 18 phoretic mites collected from nurse bees and 41 ovaries from brood mites. In phoretic mites, the somewhat larger terminal oocyte, which is

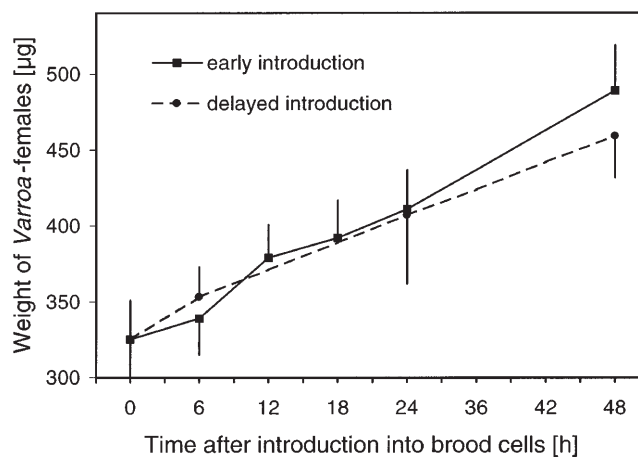


Figure 1. Increase in body weight of *V. jacobsoni* females after introduction into capped brood cells. Mites introduced immediately after capping of the brood cells had an identical weight increase as mites introduced 14 hours after capping.

expected to become the first egg, had a pale and non-specific staining after incubation in toluidine blue. It did not differ from the surrounding tissue of the ovary or the smaller oocytes (Fig. 2a). Six hours after cell sealing, a clear blue staining of the terminal oocyte was already clearly visible (Fig. 2b, Tab. I). The intensity of the blue color, however, still varied among the 18 specimens. After 12 and 18 hours, the staining of the primary oocyte was more intense and without the variation seen in oocytes sampled at 6 hours (Fig. 2c). Therefore, toluidine blue staining of the biggest oocyte, indicating uptake of euplasmatic and/or yolk material, could be verified for all 41 ovaries from brood mites (Tab. I).

From the 10 *V. jacobsoni* females introduced 14–16 h after capping of the brood cells, we could find a stained oocyte in only a single ovary, meaning that 90% of the *V. jacobsoni* mites of this group had not commenced oocyte maturation (Tab. I).

4. DISCUSSION

By the use of our new method, we were able to determine for the first time the exact timing of activation of the terminal oocyte in

Table I. Oocyte growth in dissected ovaries. *V. jacobsoni* females were introduced into freshly capped brood cells and removed at 6 hours intervals.

Time after cell capping	Number of ovaries examined	Number of activated ovaries
phoretic	18	0
6 h	18	18
12 h	11	11
18 h	7	7
24 h	5	5
24 h*	10	1

* *V. jacobsoni* females were introduced 14–16 hours after cell capping and removed 24 hours later for dissection.

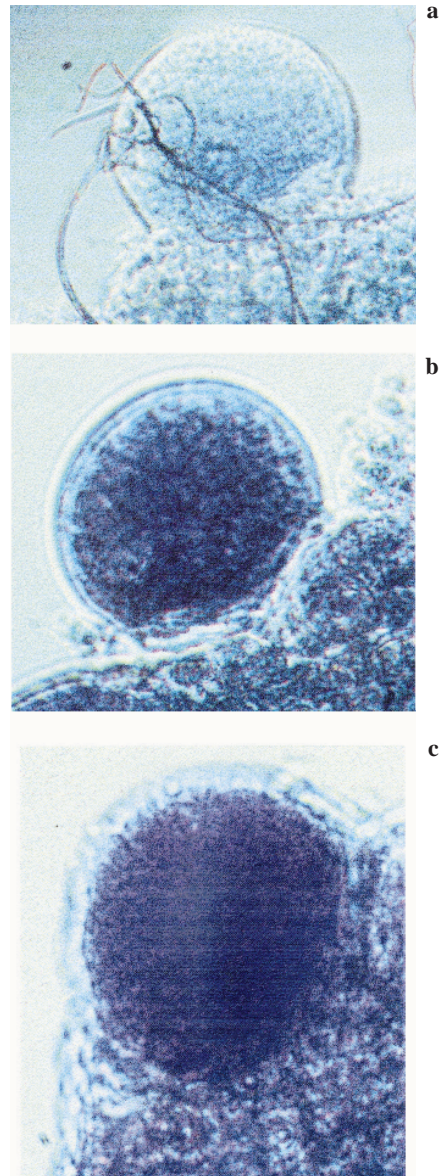


Figure 2. The terminal oocyte of *V. jacobsoni* ovaries dissected and stained according to our whole mount technique. In phoretic mites (a) the terminal oocyte is already larger than the other ones, but no incorporation of euplasmatic material or yolk protein is visible. 6 hours after cell capping (b) the first incorporation is clearly visible by blue staining. 18 hours after cell capping (c) the oocyte is completely colored.

the *V. jacobsoni* ovary. Whole mount preparations were used to examine the initiation of oocyte growth in large samples of individual *V. jacobsoni* mites. This approach can be applied even in semi-field conditions. Of course, statements about the quantity or the specificity of the material incorporated into the oocyte are not possible. For details of the course of oogenesis, time consuming sectioning and immunocytochemical techniques are still necessary [1, 22, 24].

In all the phoretic mites examined by toluidine blue staining, no coloration of the ovary was detected. Even the previously enlarged oocyte which is about to enter the first gonocycle [23] did not reveal specific staining. This confirms the view that phoretic mites remain in a previtellogenic stage [9, 23]. In our study, oocyte coloration was independent of the status of the opisthosoma of the phoretic mites ("swollen" or "not swollen" as described formerly [4]). Six hours after cell capping, the terminal oocytes already were clearly blue-colored indicating activated oocyte growth. This is significantly earlier than the assumed start of vitellogenesis, previously thought to be initiated 15 hours after cell capping [22, 23]. Probably, during this initial phase of oocyte growth, euplasmatic components supplied by the nursing lyrata organ [22] are incorporated into the oocyte rather than yolk proteins. Under the selection pressure of the relatively short postcapping period of the host [13, 18], the early start of oocyte growth may be an adaptive strategy to ensure that at least two adult descendants, one male and one female, can reach sexual maturity before the host bee emerges.

During the further course of oogenesis, additional control mechanisms may influence *V. jacobsoni* reproductive success. In all *V. jacobsoni* females which were introduced into recently sealed brood cells ($n = 41$), we confirmed activation of the ovary. Year long records at Hohenheim revealed percentages of fertile mites in worker brood of between only 80 and 90%

[16]. The lower percentage of fertile mites under natural conditions may be explained by an arrest of oogenesis in already activated ovaries, probably due to oosorption [22].

Surprisingly, *V. jacobsoni* females which were assumed to remain infertile ("delayed introduction") increase their body weight during the first 48 hours after artificial introduction to a brood cell, and to a similar magnitude as fertile mites ("early introduction"). This feeding behavior indicates the general readiness of phoretic *V. jacobsoni* females to enter the reproductive phase which, however, requires the action of an initiation factor. At least, oocyte activation does not depend on a phagostimulus or on the quantitative uptake of larval haemolymph, as has been assumed previously [20].

Our preliminary results support the hypothesis that the assumed factor for oocyte activation is only present in freshly capped brood cells. Whenever *V. jacobsoni* females are introduced into brood cells which have been capped for 14 h, they do not initiate oocyte growth at all. The nature of this hypothetical activation factor remains unknown and, since the rejection of the JH hypothesis [12, 19, 21], no other specific host-derived compound with a distinct peak shortly after cell capping has yet been identified. The very quick physiological reaction of mites supports the conclusions of Trouillier and Milani [26] that *V. jacobsoni* reproduction is stimulated by a chemotactic signal.

There is still the important question of whether the high proportion of infertile *V. jacobsoni* females in worker brood of *Apis cerana* and Africanized honey bees [14, 15], for instance, might be explained by reactions to a specific host signal. *V. jacobsoni* infertility may reflect variation of an host-dependent factor as well as a reaction of certain mite genotypes toward this signal [3, 5, 8].

Therefore, we need more data on the initiation of oocyte maturation, particularly in situations where a high percentage of infertile mites exists. Our new method offers

the possibility for exact determination of the oocyte growth of *V. jacobsoni* females and allows extensive examination in populations with different degrees of host-parasite adaptation.

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Résumé – Coloration au bleu de toluidine pour une détermination rapide du début de la croissance des ovocytes et de la fertilité chez *Varroa jacobsoni* Oud.

La fertilité réduite des acariens *Varroa jacobsoni* dans les cellules de couvain d'ouvrières (*Apis mellifera* L.) est l'un des facteurs clés liés à la tolérance à la varroose, comme cela a été montré chez l'hôte d'origine *Apis cerana* Fabr. et chez les abeilles africanisées du Brésil. Les signaux qui déclenchent l'activation de la reproduction de l'acarien après l'invasion de la cellule de couvain restent à ce jour inconnus. Nous avons utilisé une nouvelle méthode de coloration in toto pour déterminer avec précision le début de la croissance des ovocytes.

Des femelles phorétiques de *V. jacobsoni* ont été prélevées sur des abeilles adultes et introduites artificiellement dans des cellules de couvain fraîchement operculées et dans des cellules de couvain operculées depuis 14–16 h. Toutes les 6 h, les acariens ont été retirés des cellules et pesés sur une balance de précision. Immédiatement après la pesée, les ovaires ainsi que la spermathèque et l'organe en forme de lyre, ont été soigneusement disséqués, fixés dans la formaline à 4 % et colorés durant 30 min au bleu de toluidine à 0,005 %. Les ovocytes ont été lavés dans un tampon PBS et leur coloration a été analysée au microscope. La coloration de l'ovocyte dépend de l'incorporation de vitellus ou d'autres constituants euplasmatiques et marque donc l'activation de l'ovaire.

Tous les acariens présents sur le couvain ont vu leur poids augmenter d'environ 325 µg à plus de 400 µg durant les 24 h qui ont suivi l'introduction (Fig. 1), quel que soit le moment de l'introduction. Ce comportement alimentaire indique une disposition générale des femelles phorétiques de *V. jacobsoni* à entrer dans la phase de reproduction. Mais chez tous les acariens phorétiques examinés (n = 18) aucune coloration au bleu de toluidine n'a été détectée (Tab. I ; Fig. 2a). Ceci confirme les observations antérieures selon lesquelles l'ovaire des acariens phorétiques est bloqué à un stade de prévitellogenèse. Pourtant, dans les ovaires des acariens présents sur le couvain, une coloration bleu clair de l'ovocyte terminal était déjà visible 6 h après l'introduction artificielle des acariens dans les cellules fraîchement operculées (n = 41 ; Tab. I ; Fig. 2b). Sur les 10 femelles de *V. jacobsoni* introduites 14 à 16 h après l'operculation, nous n'avons trouvé un ovocyte coloré que dans un seul ovaire (Tab. I), c'est-à-dire que, chez 90 % des acariens de ce groupe, la maturation des ovocytes n'avait pas commencé.

En conclusion, la reproduction de *V. jacobsoni* doit être stimulée par un signal qui n'est présent que dans les cellules fraîchement operculées. Si les femelles de *V. jacobsoni* sont introduites dans des cellules operculées depuis 14 h, la croissance des ovocytes n'est pas du tout démarrée, ce qui indique l'absence de signal d'activation à ce stade de l'hôte. La nature du signal et les mécanismes de stimulation de la reproduction de *V. jacobsoni* restent inconnus. Notre nouvelle méthode offre la possibilité de déterminer avec précision la croissance des ovocytes dans de grands échantillons de femelles de *V. jacobsoni*. C'est donc un outil approprié pour des études en conditions semi-naturelles de la fertilité de *V. jacobsoni* dans des populations qui présentent divers degrés de tolérance à la varroose.

Varroa jacobsoni / relation hôte parasite / vitellogenèse / ovogenèse / fertilité / reproduction

Zusammenfassung – Färbung mit Toluidinblau als rasche Methode zur Beurteilung der Aktivierung von Oocytenwachstum und Fertilität bei *Varroa jacobsoni* Oud.

Eine reduzierte Fertilität der *V. jacobsoni*-Weibchen in Arbeiterinnenbrut ist einer der Schlüsselfaktoren für eine Toleranz gegenüber der Varroose. Dies wurde insbesondere durch Untersuchungen beim Ursprungswirt *Apis cerana* und bei den Afrikanisierten Honigbienen Brasiliens bestätigt. Die Signale für die Aktivierung und Steuerung der *V. jacobsoni*-Reproduktion sind aber nach wie vor unbekannt. Wir verwendeten eine neue "whole mount"-Technik, um den exakten Zeitpunkt der Oocyten-Aktivierung zu bestimmen.

Phoretische Milben wurden von Adultbienen abgesammelt und in frisch verdeckelte Brutzellen und in Brutzellen, die bereits seit 14–16 Stunden verdeckelt waren, eingesetzt. In Abständen von 6 Stunden nach dem Einsetzen wurden die Milben aus den Brutzellen entnommen und auf einer Analysenwaage das Körpergewicht bestimmt. Direkt nach dem Wiegen wurden die Ovarien zusammen mit der Spermatheka und dem lyraförmigen Organ herauspräpariert, in Formalin (4 %) fixiert und für 30 Minuten in Toluidinblau (0,005 %) gefärbt. Nach dem Waschen in PBS-Puffer wurde die Färbung der Oocyten lichtmikroskopisch bestimmt. Die Färbung der Oocyte ist mit dem Einbau von Dotter oder anderen euplastischen Bestandteilen korreliert und ist daher ein klarer Hinweis für die Aktivierung des Ovars.

Bei allen Brutmilben nahm das Körpergewicht während der ersten 24 Stunden nach Einsetzen in die Brutzellen von ca. 320 µg auf über 400 µg zu (Abb. 1). Diese Zunahme war unabhängig vom Zeitpunkt des Einsetzens der Milben. Die rasche und umfangreiche Nahrungsaufnahme lässt auf eine generelle Bereitschaft der phoretischen *V. jacobsoni*-Weibchen zur Reproduktion schließen. Allerdings wurde bei keiner der 18 untersuchten phoretischen Milben eine Toluidinblau-Färbung festgestellt (Tab. I,

Abb. 2a). Dies bestätigt frühere Untersuchungen, nach denen das Ovar von phoretischen Milben in einem prävitellogenen Stadium arretiert ist. In den Ovarien der Brutmilben wurde dagegen bereits 6 Stunden nach dem Einsetzen in frisch verdeckelte Brutzellen eine eindeutige blaue Färbung der terminalen Oocyte festgestellt (n = 41, Tab. I, Abb. 2b). Bei den 10 *V. jacobsoni*-Weibchen, die 14–16 Stunden nach der Zellverdeckelung in die Brutzellen eingesetzt wurden, konnte lediglich in einem Ovar eine gefärbte Oocyte nachgewiesen werden (Tab. I). Dies bedeutet, dass aus dieser Gruppe bei 90 % der Milben keine Aktivierung der Oocyten erfolgt war. Wir schließen daraus, dass die *V. jacobsoni*-Reproduktion durch ein Signal stimuliert wird, das ausschließlich in frisch verdeckelten Brutzellen vorhanden ist. Da die verspätet eingesetzten *V. jacobsoni*-Weibchen nicht mit der Reifung der Oocyten beginnen, ist dieses Aktivierungssignal zu diesem Zeitpunkt offensichtlich schon nicht mehr vorhanden. Unsere neue Methode erlaubt die exakte und rasche Bestimmung der Aktivierung der Oocyten bei einzelnen *V. jacobsoni*-Weibchen. Sie ist daher hervorragend geeignet zur Untersuchung der *V. jacobsoni*-Fertilität in Populationen mit unterschiedlichem Toleranzniveau.

***Varroa jacobsoni* / Wirt-Parasit-Beziehung / Vitellogenese / Oogenese / Fertilität / Reproduktion**

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