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Article

Analysis of the reproductive viability of *Varroa destructor* (Mesostigmata: Varroidae) rearing under laboratory conditions

Jesús H. Reyna-Fuentes[1], Cecilia C. Zapata-Campos[1*], Fabian Eliseo Olazarán-Santibáñez[1], José O. Merino-Charrez[1] and Daniel López-Aguirre[2]

1. *Universidad Autónoma de Tamaulipas. Facultad de Medicina Veterinaria y Zootecnia. Carretera Cd. Victoria - Cd. Mante Km. 4. Ej. Santa Librada. Ciudad Victoria, Tamaulipas. C.P. 87274. México; E-mails: cezapata@docentes.uat.edu.mx, omerino@docentes.uat.edu.mx, feolazaran@docentes.uat.edu.mx*
2. *Universidad Autónoma de Tamaulipas. Facultad de Ingeniería y Ciencias.Centro Universitario Adolfo López Mateos. Edificio Centro de Gestión del Conocimiento, 4° piso. Ciudad Victoria, Tamaulipas. C.P. 87149. México; E-mail: dlaguirre@docentes.uat.edu.mx*

[*] Corresponding author

ABSTRACT

The *Varroa destructor* Anderson & Trueman, 2000 (Mesostigmata: Varroidae) is one of the most detrimental pests for the physiology and productivity of *Apis mellifera* (Hymenoptera: Apidae) in the international context. To study its reproductive behavior, different rearing protocols have been developed over the years under semi-natural or laboratory conditions. However, *V. destructor* has low survival capacity and does not reproduce successfully outside its natural environment and hosts. As a result, the availability of these mites for experimental purposes is limited. This research aimed to evaluate the reproductive viability of *V. destructor* mites under laboratory conditions, using gelatin capsules with single and double infestation by bee pupae (5th instar). All data were analyzed using generalized linear mixed models implemented in Proc GLIMMIX using the binary distribution function with the default logit link function. For both treatments (capsule with 1 and 2 mother mites), a significant effect was shown for the mean number of broods ($p < 0.0001$). The reproduction of mother mites in both treatments showed significant effects ($p < 0.05$). Males were observed more frequently in capsules infested with two *V. destructor* mother mites ($p = 0.001$). For the case of pupae mortality with two mites a significant value of $p < 0.0007$ was reported, as well as with the presence of one mite $p < 0.0040$, however, no significant differences between them were demonstrated. In conclusion, these results showed that in vitro *V. destructor* rearing using gelatin capsules is a useful tool for future research work. However, it is intended to continue modifying this protocol to find higher values of survival and reproductive success of mites, to analyze the biological parameters of *V. destructor* in studies involving a high number of adult mites, several generations, and reproductive cycles.

KEYWORDS: Acari, bee, pupae, artificial diet, laboratory condition.

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INTRODUCTION

Honeybees (*Apis mellifera* L., 1758) play a significant role in biodiversity conservation and food supply, as they pollinate both crops and wild plant species (Abro *et al*. 2022). Additionally, they serve

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as a source of income through the sale of hive products (Gratzer *et al*. 2021), such as propolis, wax, royal jelly, pollen, and bee venom, which have various cosmetic and domestic applications (Kurek-Górecka *et al*. 2020). Recently, beekeeping has faced a global decline in honeybee populations due to colony losses (Gray *et al*. 2023). The loss of honeybees can lead to unforeseen consequences, such as the increase in pests and invasive plant species (Hristov *et al*. 2020; Bartlett 2021). Factors like climate changes affect the thermoregulation, homeostasis, growth, and survival of the colonies (Becsi *et al*. 2021; Popovska Stojanov *et al*. 2021; Cunningham *et al*. 2022). Additionally, habitat loss and pesticide exposure have contributed to the global decline in honeybee populations. However, the presence of pests and diseases is one of the most threatening stressors for *A. mellifera*. *Varroa destructor* Anderson & Trueman, 2000 mite poses the greatest threat to honeybee health, as it feeds on the host's fat body and hemolymph, consuming nearly one microliter daily (Rosenkranz *et al*. 2010; Ramsey *et al*. 2019). It also interferes with the physiology of *A. mellifera* (Arrese and Soulages 2010), as it reduces the lifespan of bees by up to 25% (Wegener *et al*. 2016). The life cycle of *V. destructor* consists of two phases: the dispersal or phoretic phase, during which it parasitizes adult bees, and the reproductive phase, which is associated with bee development (metamorphosis) (Traynor *et al*. 2020). High levels of infestation by *V. destructor* leads to decreases in the productive levels of bee colonies, and poor management of control methods can cause colony collapse within no more than two years. This mite acts synergistically with various pathogens, primarily as a vector of viruses in bee colonies (Chen *et al*. 2021). However, whether the viruses transmitted by *V. destructor* infestation and actively replicate within the mites, thereby facilitating increased transmission, is unclear (Damayo *et al*. 2023). To date, no health protocol has been able to eradicate *V. destructor* mites completely; it has only allowed to manage its population (Jack and Ellis, 2021), and this may vary based on the used compounds, as well as the methodology and management by beekeepers. To minimize the use of chemical pesticides, plant-derived products are advocated as a sustainable strategy for managing *V. destructor* infestations in beekeeping. These products contain compounds that degrade readily in the environment and are generally safe for non-target organisms, including bees (Ramzi *et al*. 2017; Roy *et al*. 2018; Potrich *et al*. 2020; Catania *et al*. 2023).

The development of an *in vitro* rearing method for *V. destructor* poses a significant challenge due to the physiological requirements of its life cycle (Rosenkranz *et al*. 2010). Since the 1980s, numerous attempts have been made to establish *in vitro* protocols. Nazzi and Milani (1994) utilized gelatin capsules containing freshly sealed bee larvae, while Bruce *et al*. (1991) developed an artificial diet based on homogenized larvae to feed mites. Although these techniques demonstrated some potential, all failed to produce reproductively mature mites in the second generation (Abbas and Engels 1988; Chiesa *et al*. 1989). Similarly, Donzé and Guérin (1997) employed controlled brood frame transfer, and Tabart *et al*. (2013) focused on developing artificial feeding chambers. However, the system developed by Nazzi and Milani (1994), used in recent studies (Piou *et al*. 2016; Annoscia *et al*. 2017; Mondet *et al*. 2018), stands out for its ability to control parameters and observe the mite's life cycle daily without altering its physiology, albeit limited to a single generation. The priority remains to optimize the reproductive success of founder mites reared in the laboratory to ensure the production of fertile daughters and achieve a functional multigenerational system. Recent studies (Vilarem *et al*. 2021) highlight advances *in vitro* and semi-*in vitro* methods developed by various authors (Bruce *et al*. 1988; Chiesa *et al*. 1989; Beetsma and Zonneveld 1992; Donzé and Guérin, 1997), emphasizing both the challenges and potential of this research area. Progress in this field would be pivotal for experiments requiring large numbers of mites, such as acaricide screening, studies of molecular resistance, and mite biology. Additionally, it would facilitate year-round research and improve reproducibility across laboratories.

The analysis of treatments involving phytochemical compounds has been ongoing, as the goal is to mitigate the negative effects of the *V. destructor* mite. Moreover, the procurement of these compounds for experimental purposes can vary depending on the regions, seasons, and climatic conditions, and it is often time consuming and can be costly (Dietemann *et al*. 2013). Therefore, the

present study aimed to evaluate the viability and reproductive behavior of the *V. destructor* mite under laboratory conditions, using gelatin capsules with single and double infestations of bee pupae (5th instar).

MATERIALS AND METHODS

Brood frames from *Apis mellifera* colonies heavily infested with *V. destructor* (infestation rates > 6%) and untreated for at least six months were collected. Phoretic infestation levels in adult bees were assessed following the de Jong (1997) method. After infestation confirmation, sealed brood frames (pupae) were transported to the laboratory for mite collection. Cells were opened using entomological forceps, and mites were collected with a fine brush and transferred to Falcon Petri dishes (50 × 9 mm). Simultaneously, brood frames containing a high proportion of fifth-instar larvae were selected. Gelatin capsules (size 7 mm external diameter; Now Foods, Bloomingdale, IL, USA) were placed over the larval cells before sealing. The frames were then arranged horizontally, with the capsules pointing down, in a 34.5 °C incubator for 2 to 5 h. The transfer of larvae in the capsules thus only relied on the spinning movements and did not involve any handling.

To evaluate the reproductive success of *V. destructor*, 90 female foundress mites were introduced into experimental treatments: 30 mites for the single-foundress group (T1; one mite per capsule) and 60 for the dual-foundress group (T2; two mites per capsule). Female mites were individually introduced into gelatin capsules containing either one (T1) or two mites (T2). Ventilation holes were created in the capsules using a size 2 insect pin (BioQuip, Rancho Dominguez, CA, USA). Capsules were placed vertically into an empty micro-pipette tip container and incubated at 34.5 °C and 75% RH until bee emergence (day 12). After 12 days, the capsules were opened and examined under a dissecting microscope. The viability of both the bee and the female mite was recorded, along with the presence, number, and developmental stages of mite offspring.

Data analysis

All data were analyzed using generalized linear mixed models implemented in Proc GLIMMIX (SAS/STAT v.14.1; SAS Institute, Cary, NC, USA) with the default logit link function and binary distribution. Survival and reproduction proportions were analyzed as binary variables (with Yes/No responses), while brood data were treated as count data. The response variables included: *Varroa* sp. offsprings, presence of males in the capsule, number of daughters (*Varroa*) per capsule, and pupal (bee) mortality.

RESULTS

After confirming that the response variables did not meet the assumptions of normality (Shapiro-Wilk test, Table 1) or homoscedasticity (Table 2), the offspring production of *V. destructor* mites was analyzed using Poisson distribution models for single and double infestation treatments. For these analyses, a total of 90 foundress mites were introduced: 60 mites were assigned to the two-foundress-per-capsule treatment, while 30 mites were assigned to the one-foundress-per-capsule treatment. The results revealed a significant effect of infestation treatment on the mean number of offspring produced ($p < 0.0001$). The proportion of capsules in which reproduction occurred also differed significantly between treatments: in the two-foundress-per-capsule group, 56.66% of the capsules produced offspring, yielding a total of 51 offspring, whereas in the one-foundress-per-capsule group, reproduction was observed in 6.66% more capsules than expected based on the number of mites introduced, producing 32 offspring (GLMMIX $\chi^2 = 3.76$; df = 1; $p < 0.05$). However, no statistically significant differences in the total offspring numbers were detected between the two treatments (Table 3).

Table 1. *Varroa destructor* mite offspring, through single and double infestation (Poisson model).

Response variable (No. of mites)	Mean	SEM	*P value*
1	1.40[b]	0.01	< 0.0001
2	2.02[a]	0.01	< 0.0001

Means in each column with different literals are significant ($p < 0.05$). SEM. Standard error of the sample mean.

Table 2. Evaluation of pupae mortality with two treatments.

***Apis mellifera* pupae mortality**			
Response variable (pupa alive)	**Mean**	**SEM**	***P value***
1 mite	0.73[a]	0.03	0.0040
2 mite	0.86[a]	0.02	0.0007
Pupa without mites	0.40[b]	0.04	0.09
Odds Ratio			3.25
χ^2			0.0003

Means in each column with different literals are significant ($p < 0.05$). SEM. Standard error of the sample mean.

Table 3. Reproductive evaluation of *V. destructor* with one or two mother mites.

Foundress mite reproduction			
Response variable (foundress mite)	**Mean**	**SEM**	***P value***
1 mite	0.80[b]	0.05	0.016
2 mites	0.93[a]	0.03	0.009
Odds Ratio			3.50
χ^2			0.14

Means in each column with different literals are significant ($p < 0.05$). SEM. Standard error of the sample mean.

The mortality rate of *Apis mellifera* pupae was significantly affected by the presence of *V. destructor* mites. Specifically, pupa mortality rates were significant in the presence of two mites ($p < 0.0007$) and one mite ($p < 0.0040$); however, no significant differences were observed between these two treatments. In contrast, the absence of mites did not result in a significant pupa mortality rate ($p > 0.05$). The analysis indicated that the presence of one or two *V. destructor* mites increases the likelihood of pupa mortality to more than 30% (32.5%; 95% CI: 3.2, 1.1–6.1) (GLIMMIX $\chi^2 = 0.0003$; df = 6; $p = 0.0014$). This difference in pupa survival can be attributed to the increased competition within the capsule environment when more than one mite is present, leading to a higher probability of pupa mortality in the natural setting of these organisms (Table 4).

The reproduction of foundress mites was significantly influenced in both treatments ($p < 0.05$). However, no significant differences were observed in the proportion of reproduction between treatments (GLMMIX $\chi^2 = 0.14$; df = 4; $p > 0.05$). Notably, the introduction of two mites per capsule increased the probability of observing reproduction within the cells by 35% (35%; 95% CI: 3.5, 0.64–18.97) by the end of the 12-day reproductive cycle (Table 3).

The presence of male mites serves as an indicator of the relationship between mating and offspring production. The results reveal that male mites are observed significantly more frequently in capsules infested with two *Varroa destructor* foundress mites ($p = 0.001$). Furthermore, the proportion of male mites differs significantly between treatments (GLMMIX $\chi^2 = 0.0001$; df = 1; $p = 0.0191$). The addition of two founding mites per capsule increases the probability of successful reproduction *in vivo* by over 30% (32.5%; 95% CI: 0.88–11.89).

Table 4. Presence of male *V. destructor* in gelatine capsules.

Male mite presence			
Variable (male mite)	**Mean**	**SEM**	***P value***
1 mite	0.66[b]	0.04	0.018
2 mite	0.86[a]	0.02	0.001
Odds Ratio			3.25
χ^2			0.07

Means in each column with different literals are significant ($p < 0.05$). SEM. Standard error of the sample mean.

DISCUSION

This study provides a foundation for the development of future in vitro rearing protocols for *Varroa* spp. mites. In Mexico, it is the first study to analyze the reproduction of *Varroa destructor* under laboratory conditions, comparing single and double mite infestations on *Apis mellifera* pupae. Initially, the reproduction of this mite was analyzed in 7 mm gelatin capsules, where the addition of two *V. destructor* founding mites resulted in greater offspring ($p < 0.0001$). Nazzi and Milani (1994) reported similar results using the same capsule size. However, they described the average oviposition of founding mites as 3.5 eggs per capsule, which differs from the present study, where the average offspring in double-infested capsules was 1.97 mites, and 1.32 in capsules with a single founding mite. On the other hand, Piou and Vétillard (2020) reported differences between single and double infestations. The mean offspring per founder was higher in capsules infested with two parasites (2.74 ± 0.15) than in those infected with a single parasite (2.23 ± 0.21). These results are similar to those of the present study, as the rearing conditions were similar. Similarly, Jack *et al*. (2020) reported *in vitro* rearing of *V. destructor* using 6, and 7 mm gelatin capsules with relative humidities of 35%, 65%, 75%, and 85%. The 7 mm capsules with 75% RH stood out in terms of mite survival and offspring. Multiple infestations of *A. mellifera* larval cells by *V. destructor* are common, with up to six founding mites found in a single worker or drone cell (Eguaras *et al*. 1994; Martin 1995). However, Anderson and Fuchs (1989) and Boot *et al*. (1997) describe decreases and increases in the number of daughters per female in the presence of a second mite. On the other hand, Donzé *et al*. (1997) reported results similar to those presented in this study, where the number of daughters was higher in cells infected with two mites (1.07) compared to cells infested individually (0.83).

Additionally, the reproduction of *V. destructor* mites is associated with the survival of *Apis mellifera* pupae, as this mite is heavily dependent on bee reproduction (Rosenkranz *et al*. 2010). Consequently, the survival rate and offspring of *V. destructor* increased as *A. mellifera* larvae survived. The mortality rate of bee pupae did not show significant differences when a second mite was introduced into the capsules. This finding aligns with Piou and Vétillard (2020), who reported mortality rates ranging from 84.3% to 98.8% with the addition of two founding mites. However, mortality rates with the presence of a single mite ranged around 87%. Mortensen *et al*. (2018) and Mortensen *et al*. (2023) noted that bees reared *in vitro* exhibit physiological and morphological behaviors different from naturally reared bees. Therefore, it is concluded that bee pheromones significantly influence mite behavior (Calderone and Lin 2001; Aumeier *et al*. 2002; Nazzi and Le Conte 2016). The proportion of capsules in which reproduction was initiated was significantly different between the two infestation treatments. The present results are consistent with those described by Piou and Vétillard (2020), who reported a 94.3% reproduction rate of founding mites with the addition of two mites and a 75.9% reproduction rate with the presence of a single mite. The reproductive parameters observed with one mite per cell were similar to those reported by Muntaabski *et al*. (2023), where a 79.6% fertility rate of founding mites was documented. Similarly, Häußermann *et al*. (2020) described fertility ranges of 75–95%. These results align with the natural reproductive behavior of *V. destructor* in temperate climates (Locke and Fries 2011; Frey *et al*. 2013). On the other

hand, they differ from those described by Beetsma and Zonneveld (1992), who reported mite fertility rates below 24%. In conclusion, the addition of one mite in the capsules increases the likelihood of observing reproduction in the cells at the end of the cycle (12 days). Therefore, the fertility rate of the mite could be influenced by the physiological state of *A. mellifera* pupae, due to the release of pheromones, which indicates an interaction between the optimal physiological state for the reproduction of *V. destructor* (Kirrane *et al*. 2011).

Finally, an interaction between the reproduction of the founding mite and the proportion of males in the capsules is demonstrated. The results show significant differences between the treatments, with males being observed more frequently in capsules with double mite infestation. This is consistent with the findings of Piou and Vétillard (2020), who reported male mite presence ranges of 61.7–86.2% with the addition of two founding mites. However, for capsules with a single infestation, our findings differ from those authors, who reported ranges below 52%. In conclusion, the percentage of capsules containing males on day 12 was higher in the case of double infestation compared to single mite infestation, indicating a higher proportion of fertilized mites. Thus, we confirm that the number of mated mites per female is also affected by the low survival rate of male offspring, which is similar to the survival rate of males in Africanized bees (Medina and Martin 1999).

CONCLUSION

This research provides information for the methodological development of *V. destructor* mite rearing, aiming to obtain experimental mites with greater ease, especially during periods when they are difficult to find. These results demonstrate that in vitro rearing of *V. destructor* using gelatin capsules is a useful tool for future research, with potential for additional modifications such as the management of relative humidity and the addition of substrates that enhance the viability of *A. mellifera* larvae, among others. Furthermore, it is concluded that artificially infesting the capsules with two mites increases the number of mated females per founding mite, due to the probability of having at least one live founding mite that sustains the offspring. In conclusion, the recorded survival and reproductive success rates of the mites indicate that this rearing method is suitable for analyzing the biological parameters of *V. destructor* in studies involving a large number of adult mites, multiple generations, and reproductive cycles.

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تجزیه و تحلیل زنده‌مانی تولیدمثلی *Varroa destructor* (Mesostigmata: Varroidae) پرورش یافته در شرایط آزمایشگاهی

خسوس اچ. رینا-فوئنتس[1]، سیسیلیا سی. زاپاتا-کامپوس[1*]، فابیان الیزئو اولازاران-سانتیبانز[1]، خوزه او. مرینو-چارز[1] و دانیل لوپز-آگویر[2]

۱. دانشگاه خودمختار تامائولیپاس. دانشکدهٔ دامپزشکی و دامپروری. کیلومتر ٤ بزرگراه شهر ویکتوریا به شهر مانته. سانتا لیبرادا. شهر ویکتوریا، تامائولیپاس کد پستی ۸۷۲۷٤ مکزیک؛ رایانامه‌ها: cezapata@docentes.uat.edu.mx، omerino@docentes.uat.edu.mx، feolazaran@docentes.uat.edu.mx

۲. دانشگاه خودمختار تامائولیپاس. دانشکدهٔ مهندسی و علوم. مرکز دانشگاهی آدولفو لوپز ماتئوس. ساختمان مرکز مدیریت دانش، طبقه چهارم. شهر ویکتوریا، تامائولیپاس، کد پستی ۸۷۱٤۹ مکزیک؛ رایانامه: dlaguirre@docentes.uat.edu.mx

* نویسنده مسئول

چکیده

هرنای *Varroa destructor* Anderson & Trueman, 2000 (Mesostigmata: Varroidae) یکی از مضرترین آفات برای فیزیولوژی و بهره‌وری *Apis mellifera* (Hymenoptera: Apidae) در سطح بین‌المللی است. برای مطالعه رفتار تولیدمثلی آن، شیوه‌نامه‌های پرورشی مختلفی در طول سال‌ها در شرایط نیمه طبیعی یا آزمایشگاهی توسعه داده شده است. با این حال، *V. destructor* ظرفیت زنده‌مانی کمی دارد و در خارج از محیط طبیعی و میزبان‌های خود با موفقیت تولیدمثل نمی‌کند. در نتیجه، دسترسی به این هرناها برای اهداف آزمایشی محدود است. این پژوهش با هدف ارزیابی قابلیت تولیدمثل هرنای *V. destructor* در شرایط آزمایشگاهی، با استفاده از کپسول‌های ژلاتینی با آلودگی تکی و دوتایی شفیره‌های زنبور عسل (سن پنجم) انجام شد. تمام داده‌ها با استفاده از مدل‌های ترکیبی خطی تعمیم‌یافته که در Proc GLIMMIX با استفاده از تابع توزیع دوگانه با تابع پیوند لوجیت پیش‌فرض پیاده‌سازی شده بودند، تجزیه و تحلیل شدند. برای هر دو تیمار (کپسول با ۱ و ۲ هرنای مادر)، اثر معنی‌داری بر میانگین تعداد نوزادان نشان داده شد ($p < ۰/۰۰۰۱$). تولیدمثل هرناهای مادر در هر دو تیمار اثرهای معنی‌داری را نشان داد ($p < ۰/۰۵$). نرها بیشتر در کپسول‌های آلوده به دو هرنای مادر *V. destructor* مشاهده شدند ($p = ۰/۰۰۱$). برای مورد مرگ و میر شفیره‌ها با دو هرنا، مقدار معنی‌داری $p < ۰/۰۰۰۷$ گزارش شد، همچنین با وجود یک هرنا $p < ۰/۰۰٤۰$، با این حال، هیچ تفاوت معنی‌داری بین آنها نشان داده نشد. در نتیجه، این نتایج نشان داد که پرورش *V. destructor* در شرایط آزمایشگاهی با استفاده از کپسول‌های ژلاتینی ابزاری مفید برای کارهای پژوهشی آینده است. با این حال، هدف این است که به اصلاح این شیوه‌نامه ادامه داده شود تا مقادیر بیشتری از زنده‌مانی و موفقیت تولیدمثلی هرناها پیدا شود، تا فراسنجه‌های زیستی *V. destructor* در مطالعاتی که شامل تعداد زیادی هرنای بالغ، در چندین نسل و چرخه‌های تولیدمثلی هستند، تجزیه و تحلیل شود.

واژگان کلیدی: کنه، زنبور عسل، شفیره، غذای مصنوعی، شرایط آزمایشگاهی.

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