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Noble I. Egekwu

Francisco Posada

Daniel E. Sonenshine
*Old Dominion University*

Steven Cook



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# Using an in vitro system for maintaining *Varroa destructor* mites on *Apis mellifera* pupae as hosts: studies of mite longevity and feeding behavior

**Noble I. Egekwu[1] · Francisco Posada[1] · Daniel E. Sonenshine[1,2] · Steven Cook[1]**

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**Abstract** *Varroa destructor* mites (varroa) are ectoparasites of *Apis mellifera* honey bees, and the damage they inflict on hosts is likely a causative factor of recent poor honey bee colony performance. Research has produced an arsenal of control agents against varroa mites, which have become resistant to many chemical means of their control, and other means have uncertain efficacy. Novel means of control will result from a thorough understanding of varroa physiology and behavior. However, robust knowledge of varroa biology is lacking; mites have very low survivability and reproduction away from their natural environment and host, and few tested protocols of maintaining mites in vitro are available as standardized methods for varroa research. Here, we describe the 'varroa maintenance system' (VMS), a tool for maintaining in vitro populations of varroa on its natural host, and present best practices for its use in varroa and host research. Additionally, we present results using the VMS from research of varroa and host longevity and varroa feeding behavior. Under these conditions, from two trials, mites lived an average of 12 and 14 days, respectively. For studies of feeding behavior, female mites inflicted wounds located on a wide range of sites on the host's integument, but preferred to feed from the host's abdomen and thorax. Originally in the phoretic-phase, female mites in VMS had limited reproduction, but positive instances give insights into the cues necessary for initiating reproduction. The VMS is a useful tool for laboratory studies requiring long-term survival of mites, or host–parasite interactions.

**Keywords** Acari · Defecation · Gelatin capsule · Reproduction

✉ Noble I. Egekwu
noble.egekwu@ars.usda.gov

[1] Bee Research Lab, USDA-ARS, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA

[2] Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529, USA

## Introduction

*Varroa destructor* Anderson and Truman (Acari: Varroidae) (varroa), an ectoparasite of the European honey bee *Apis mellifera* (honeybee) has been implicated as a causative factor in the general decline in honey bee colony health (Fazier et al. 2010; Nazzi and Le Conte 2016). Varroa mites cause a range of harmful effects on their hosts; for example, varroa mites transmit a number of honeybee viruses (Dainat et al. 2012; McMenamin and Genersch 2015). Several studies have shown a correlation between this obligate parasite and viral load of the deformed wing virus (DWV), the Israeli acute paralysis virus (IAPV), and other less well-known viruses (Chen et al. 2004; Di Prisco et al. 2011a, b; Yang and Cox-Foster 2007). Additionally, varroa causes direct physical damage/deformities from feeding (Kanbar and Engels 2003), compromises host immune systems (Koleoglu et al. 2017), and increases host mortality (Guzman-Novoa et al. 2010). As a harmful pest of agriculturally important honey bees, varroa poses a tremendous threat to the bee-keeping industry (Gibbs and Muirhead 1998; Ellis et al. 2010), and thus food security.

Rearing colonies and strains of model external parasites is critical to studying their biology and behavior as well as for developing tools for integrated pest management (IPM) strategies. Studies of host–parasite interactions and biology of parasites are severely limited by dependence on a number of key factors, including seasonal availability of hosts and their parasites, knowledge of past and present physiological status of parasites, survival and successful reproduction of parasites within a very limited range of environmental conditions, and failure of parasite to adapt to artificial diets (Lourenço and Palmeirim 2008). Varroa is an obligate ectoparasite of honey bees and thrives exclusively in honey bee hives. Thus, the particular biological requirements for sustaining varroa life in the laboratory may be a factor for the dearth of published standardized protocols for maintaining varroa in vitro. Additionally, varroa mites have a peculiar life cycle that is characterized by two separate physiological life stages; the phoretic and foundress stages. Phoretic female mites use adult honeybee host as vehicles to locate a suitable host (brood cell nearing capping with wax) for its reproductive phase. Once a phoretic mite locates and enters a cell, it enters the foundress stage. Soon after the capping of the hosts' cells, the foundress begins to lay eggs.

Varroa reproduction in vitro, specifically past the F1 generation, continues to be a challenge to researchers. Moreover, a standard protocol for maintaining varroa in the laboratory free of honey bee hosts would provide a ready source of mites of known age and history for research purposes, free from seasonal limitations. Nonetheless, successful maintenance of mites together with live hosts will provide much needed insights on the parasite's role as a vector of viral pathogens to bees, as well as knowledge of their biology (Conte et al. 2010; Rosenkranz et al. 2010; Smith et al. 2013; van Engelsdorp and Meixner 2010). Although several studies have attempted to establish a varroa rearing system (Nazzi and Milani 1996), the results of such new knowledge obtained from intensive study using mites from our maintenance system can lead to an improved rearing program which may be leveraged to quicken the search for novel means of control.

Toward this end, we describe here a novel system, the 'varroa maintenance system' (VMS), for maintaining populations of varroa mites in vitro, and also describe experiments that demonstrate how it may be a tool for understanding behavioral and physiological aspects of varroa biology. The aims of these experiments were to (1) provide best practices for maintaining varroa mites in the laboratory on their natural host, (2) extend mite longevity in vitro, and (3) better understand mite feeding behavior. Results from this study will

provide crucial information needed for the next step of designing and developing a truly artificial varroa rearing system which will provide abundant laboratory-reared mites outside of the hive with known age and physiological status.

## Materials and methods

### System design

The VMS as shown in Fig. 1 includes components readily accessible to research laboratories, and is designed to mimic structural substrates and environmental conditions within a honey bee hive, the natural environment of varroa mites. Brood host cells are represented as clear Veg K-Caps size 00 capsule (Capsuline, Pompano Beach, FL, USA) gelatin capsules, which are inserted into a 32/8-Place Combo Tube Rack (USA Scientific, Orlando, FL, USA) designed to hold 30 gel capsules with the bottom half way inward and lid outward. Unlike other similar studies (Nazzi and Milani 1996; Trouiller and Milani 1999; Piou et al. 2016), the capsules were without perforations.

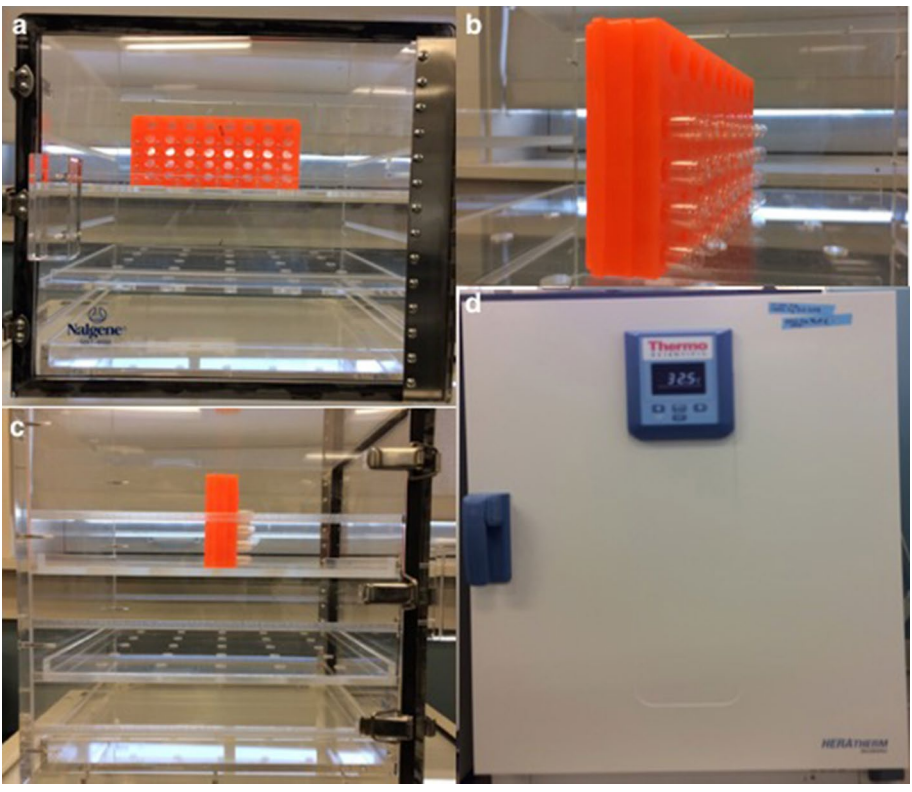


**Fig. 1** The 'varroa maintenance system' (VMS) within a VMS-chamber showing a rack with gelatin capsules. This system simulates a bee hive

Together, capsules and rack represent a 'frame' of brood comb. Multiple 'frames' may be stored in a VMS-chamber designed from a Nalgene Acrylic Desiccator Cabinet (Thermo Fisher Scientific, San Jose, CA, USA) with compartments divided by two transparent perforated trays. The perforations in these two trays allow free circulation of gases when the cabinet door is closed. A third tray (depth) without perforations is located at the bottom. This latter tray may be filled with saturated solution of potassium nitrate (Sigma-Aldrich, St Louis, MO, USA) to provide a stable relative humidity (RH) of $\geq 85\%$. The VMS-chamber may be placed in a temperature-controlled incubator, and together these simulate the physical conditions inside a natural honey bee colony.

Our system for maintaining varroa mites in vitro is described herein. This system was designed using information drawn from previous research of varroa biology (Chiesa et al. 1989; Dietemann et al. 2013; Nazzi and Milani 1994), and was modified over many iterations to determine environmental conditions that best promoted varroa and host longevity. With the results from these trials, we established best practices for handling honey bee hosts and mites when using this system.

## Best practices

Three experiments—trial 1, trial 2, and a control—were run to determine longevity of varroa in the VMS. The environmental conditions were kept at $32.4 \pm 0.1$ °C and $87.2 \pm 2.6\%$ RH. The trials determining best practices were conducted using honey bee pupae and varroa mite specimens obtained from an apiary located at USDA-ARS Beltsville Agricultural Research Center (Beltsville, MD, USA). The apiary contained bee hives that were dedicated to mite research, and thus were not treated with chemicals for varroa control. The VMS at 32.5 °C and 85% RH is most conducive to maintaining healthy hosts and parasites.

### *Harvesting and handling* Apis mellifera *pupae*

White- or pink-eyed worker pupae were collected randomly from capped cells of frames obtained from designated colonies. Each cell was uncapped with a Dumont #5 forceps (Fine Science Tools, Foster City, CA, USA). Caution was taken to avoid any form of injury to the pupae. Each pupa was carefully lifted with a pair of 0.13-cm narrow-tip featherweight forceps (Bioquip, Rancho Dominguez, CA, USA) from the exposed brood cell and deposited into a clear gelatin capsule. Pupae damaged or dropped during removal were discarded. To determine pupa quality, each pupa was checked for traces of cuticular melanization, indicating a constitutive immune response to injury in these insects (Lambrechts et al. 2004; Lee et al. 2008; Prokkola et al. 2013). Any pupae found with this condition were discarded.

### *Harvesting and handling* Varroa destructor *mites*

Phoretic-phase (i.e., not inside capped cells) varroa females were collected from nurse bees on frames retrieved from designated honey bee colonies. To do so, first, a frame was chosen at random from hives at a designated apiary. Next, honey bees were gently swept off the frames into a wide plastic basin with a bee brush. Varroa mites are removed from collected nurse bees using a modified sugar-shake method (Fakhimzadeh 2001). First, approximately 32.5 mg of confectioner sugar powder (Domino Foods, Yonkers, NY, USA) was measured into a wide-mouth 500-ml Mason jar having a wire-meshed lid. Approximately 150–250

honey bees collected from a frame were accumulated into the jar containing sugar powder. The jars were agitated till the bees were completely covered in sugar powder and allowed to sit for about 3 min. Next, varroa females were sieved out along with the sugar powder onto a shallow pan containing room temperature water. The water was drained through a miniature sieve supported with a handle to collect varroa mites. Finally, the mites were transferred onto a wet paper towel placed in a large petri dish with a lid, and immediately transported to the laboratory. It is important to have all equipment present on site during varroa collection to reduce the introduction of any form of stress that may compromise quality of experimental mites. Mites were observed for activity and the most robust, active individuals were collected using a fine paintbrush for use in the VMS; inactive, sluggish or moribund mites were discarded. After the hosts were assembled into 'cells' for experiments, mites were placed on hosts by using a soft paintbrush and allowing the mites to transfer to the pupal hosts. Forceps are strongly discouraged when handling mites.

## Experiments

For all experiments, honey bee pupae and varroa mites were collected from colonies at apiaries, using the methods described in the previous section. Additionally, only early stage pupae, specifically white- and pink-eyed developmental stages were used as hosts. To infest a honeybee host, a single female mite was 'let off' onto each pupa with a paintbrush, and the lid to the capsule housing pupa was replaced. The capsule was inserted halfway into the rack. Each VMS was made up of racks that held 32 capsules, each with a honeybee pupa host and a varroa female (Figs. 1, 2). For a control, a VMS was prepared to hold 30 mite capsules. Each mite was introduced into individual gelatin capsules without honeybee pupa host as described in de Guzman et al. (1993). RH $\geq$ 85% was achieved with saturated nitrate solution (Rockland 1960; Wexler and Hasegawa 1954).

The VMS was transferred into the VMS-chamber and the door was shut. The VMS-chamber was placed in a Heratherm Compact Microbiological Incubator (Thermo Scientific). Growth conditions for this study were $32.4 \pm 0.1$ °C and $87.2 \pm 2.6$% RH. A Traceable Thermometer/Hygrometer (Control Company, Galveston, TX, USA) was used to measure and monitor the temperature and RH during the experiments. Female mites were maintained individually on a single pupa host in all capsules in the VMS. Over the course of experiments, daily observations of the physical condition of both mite and host, location of mite on host, and other notable observations were recorded.

### Mite longevity on natural host

During the course of the experiment, which ran for 17 and 33 days for trials 1 and 2, respectively, honey bee pupae were monitored once daily by physical observation for signs of necrosis and other deformities. Developmental changes of hosts were also monitored and recorded. Any pupa observed to deteriorate physically was replaced with a fresh specimen. Any pupa that developed into an adult was considered emergent. In a case where a mite was still alive with a dead host, a fresh pupa was provided. Melanization, mechanical damage, shrinking, and water loss are characteristic of a poorly developing pupa and were checked regularly to avoid early varroa mortality that might arise from a depleting food source. In order to monitor the mites, the VMS was taken out of the VMS-chamber,

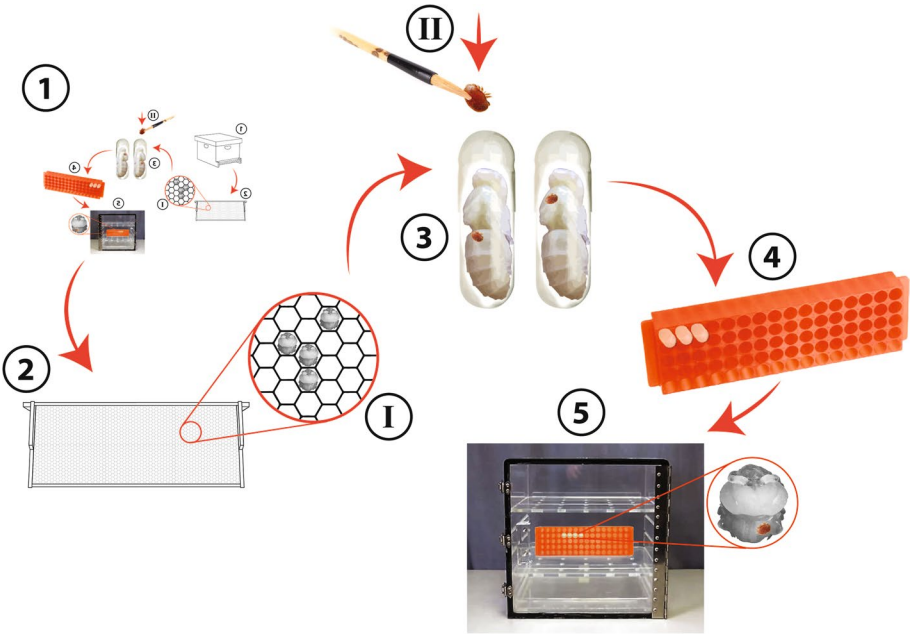


**Fig. 2** Workflow of experimental setup showing 'varroa maintenance system' (VMS). (**1**) Bee hive. (**2**) Frame of capped brood, with capped cells (I). (**3**) Capsules, mimicking capped cells, housing *Apis mellifera* pupae infested with *Varroa destructor*. (**4**) Rack, holding capsules to mimic a frame (as in 2). (**5**) VMS in VMS-chamber, mimicking a bee-hive. (II) Paintbrush with a varroa mite

observed and returned as quickly as possible. Mites that failed to move when prodded with a paintbrush were treated as dead and removed from the experiment.

## Mite feeding site preference

After pupae were exposed to varroa and upon death of the mite, pupae were incubated at room temperature overnight in 0.01% Trypan blue (Gibco Laboratories, Chagrin Falls, OH, USA) and then transferred into 70% ethyl alcohol to preserve the specimens. The pupae were transferred on a Petri dish and examined with a Stem SV 11 stereoscope fitted with an Axio Cam MRC camera (Carl Zeiss, Stockholm, Sweden). The AxioVision imaging software (Carl Zeiss) was used to visualize the wounds. Based on observations taken from preliminary trials, and for feeding preference studies reported here, a coding system was adopted to categorize the parts of the pupa where feeding holes could be located. The codes were as follows: head (H), between head and thorax (HT), thorax (T), between thorax and abdomen (AT), abdomen (A), and legs (L) (Fig. 3).

## Statistical analysis

Survival analysis and statistics were performed using Microsoft Excel and JMP Software (SAS, Cary, NC, USA) programs. Varroa mortality data were evaluated and analyzed by Wilcoxon test. Tukey's honestly significant differences (HSD) post hoc test was applied to

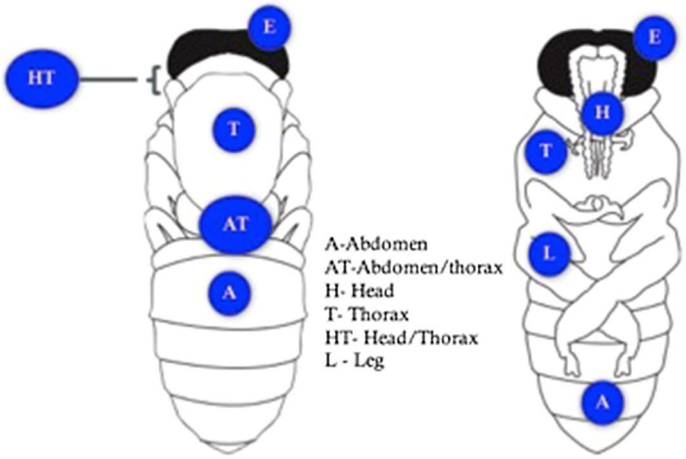


**Fig. 3** *Apis mellifera* coded for feeding sites of mites: abdomen (A), between abdomen and thorax (AT), head (H), thorax (T), between head and thorax (HT), and leg (L)

compare mean survival times ($\alpha = 0.05$). The duration for 50% survival of pupae and mites was determined from the survival curve. Feeding site preference of the mites on their hosts was calculated from daily observations.

## Results and discussion

### Mite survival

The VMS is a simple system that was designed to support varroa survival to accommodate nearly any experimental assay specifically involving the honeybee ectoparasites. The survival data of varroa females reared with this system is presented in Fig. 4. The results showed that varroa mean (±SE) survival time was significantly enhanced by the VMS (trial 1: 12.23 ± 0.54 days [n = 52]; trial 2: 14.19 ± 1.27 days [n = 51]; control: 1.07 ± 0.05 days [n = 30]; Wilcoxon test: $\chi^2 = 125.50$, df = 2, $p < 0.001$). The median survival times were 13 and 9 days for trials 1 and 2, respectively. A pairwise contrast of the varroa mean survival times using the post hoc Tukey HSD test indicated that control vs. trial 1 and control vs. trial 2 were significantly different, but not trial 1 vs. trial 2. 50% of varroa survived after 12 days in trial 1 and after 12.5 days in trial 2, when maintained with the VMS. This means that the ability or effectiveness of the VMS to maintain healthy varroa mites is strongly dependent on the presence and quality of a honeybee pupa host.

We observed that changing and replacing pupa hosts every 6 days irrespective of hidden damages not only extended mite survival, but also ruled out bias that an injured pupa might bring to the experiment as a result of poor-quality host. Also, using L5 larvae/prepupae may further extend this period, as varroa life cycle and reproductive biology interplay with honeybee host pupation. However, pupae at this specific developmental stage are ephemeral, and extremely susceptible to mechanical damage. Clearly, honeybee pupae provided support for varroa survival as also mentioned in Loftus et al. (2016). A number of hosts completed pupation (ca. 7–14 days; Fig. 5), emerging as functional adult workers,

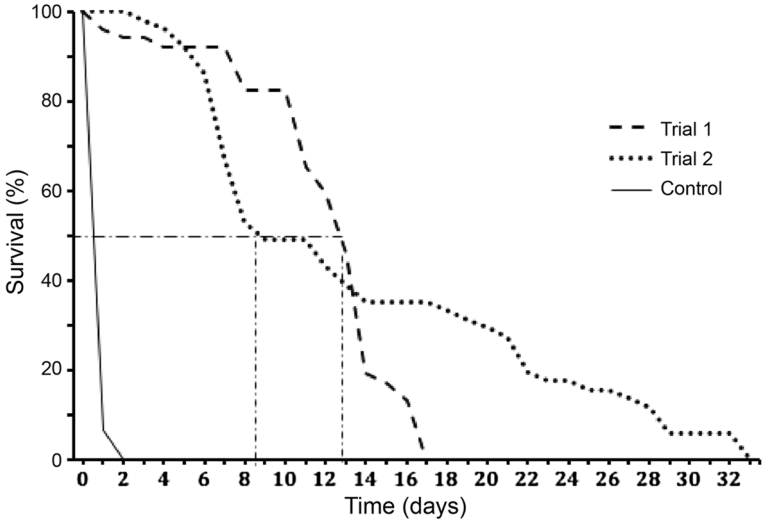


**Fig. 4** Survival (%) over time (days) of adult *Varroa destructor* females after artificial infestation on honey bee pupa hosts maintained in the varroa maintenance system. In trials 1 and 2 a bee pupa was present in each cell, in the control it was not. Some pupa hosts were replaced after 5–6 days and others were allowed to emerge to adults before replacement

suggesting physical conditions of the VMS were conducive to proper host development. Figure 5 shows the development of a healthy pupa infested with varroa during an experimental run. Because both host and parasite survive through host pupation, the system can be a useful tool for research of long-term host–parasite interactions between honey bees and varroa mites.

Although a few studies have demonstrated success in maintaining varroa mites in vitro using Petri dishes, gelatin capsules, and various substrates (Chiesa et al. 1989; de Guzman et al. 1991; Donze and Guerin 1994), most studies were conducted using foundress mites, actual honey bee hives, or were designed for the specific purpose of studying mite reproduction. In this study, however, we focused on longevity of varroa on its natural host within the laboratory and in so doing, present other interesting behavioral attributes observed and worthy of exploring. In addition, we show that the VMS is able to maintain mites longer than any other studies have shown. We present a successful system to study mite longevity with a wide range of downstream applications in the study of varroa. It is also worthy to note that this system is presently in use for other studies involving varroa.

## Feeding preference

Varroa females have serrated mouthparts that may be adapted for cutting through the cuticle of the honeybee host (Griffiths 1987). Using their mouthparts, mites create relatively large feeding holes in a host's integument, and these wounds have delayed healing (Donze and Guerin 1994; Herrmann et al. 2005; Kanbar and Engels 2003, 2004). Varroa mites maintained on a honey bee pupa may feed from a single hole or multiple feeding holes (Kanbar and Engels 2004, 2005). Upon vital staining of honeybee pupae with Trypan blue solution, these wounds become visible (Herrmann et al. 2005; Kanbar and Engels 2004). Figure 6 shows photographs taken during our observations of stained

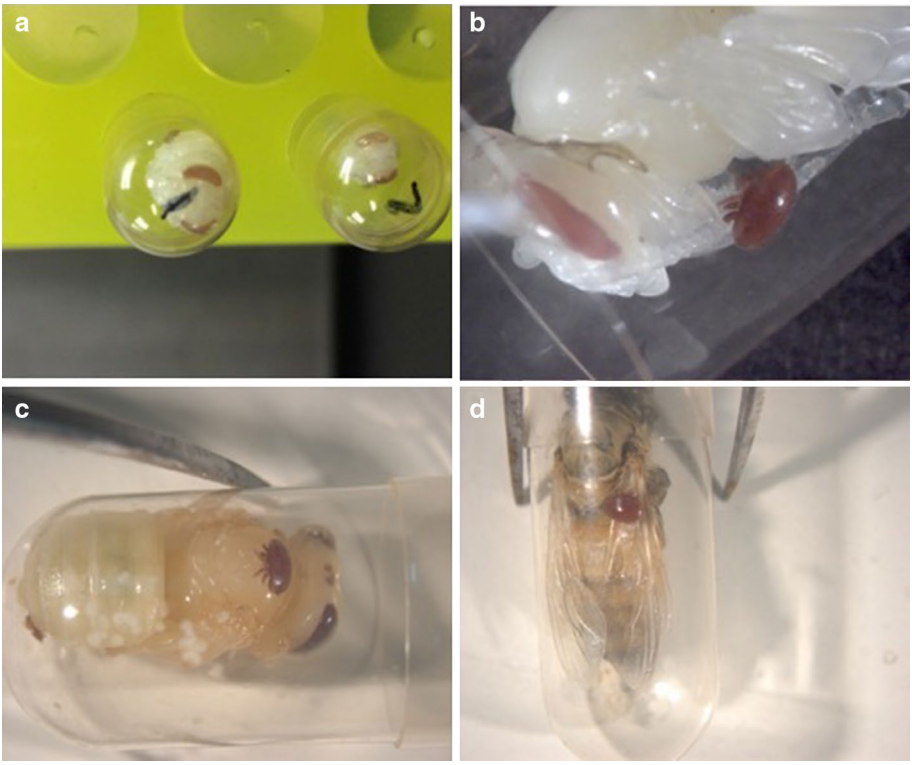


**Fig. 5** Progression of pupa quality and development during infestation with female *Varroa destructor.* **a, b** Healthy honeybee pupa fresh from capped brood cell without signs of melanization or wounds; **c** honeybee dark-eyed pupa changing coloration to light brownish, shows evidence of fecal deposition activity (white specs) on wall of capsule and body of pupa; also, evidence of successful feeding of mite on host; **d** honeybee adult emerging with wings indicating bee survival despite varroa parasitism

wounds on the head, thorax, between the head and thorax, abdomen, and legs of honeybee pupa. These observations indicate that varroa can feed from diverse locations on its host, a phenomenon we call 'substrate site dynamism' as illustrated in Fig. 7. Flexibility in location of feeding site may represent an adaptive strategy by varroa for when inside crowded cells, to gain access to food for their nutritional needs on any part of the host even when trapped by host appendages.

Varroa mites do show a feeding site preference. Based on the assumption that location of mites may indicate feeding site preference, Fig. 7 shows the highest frequency of varroa 'preferred feeding sites' (PFS) was found on the abdomen (25%, 764 PFS) and the thorax (14%, 420 PFS); the least preferred were the head (4%, 110 PFS) and between head and thorax (1%, 41 PFS).

A preference of varroa for feeding from their host's abdomen corresponds with previous studies that documented varroa females appearing to prefer to feed from their host's second abdominal segment (Fig. 6a), taken from observations that more time was spent on this body part than others anytime the mites were on their pupa host, but also from observations that varroa did not show particular preference for any body part while on the prepupae (Calderon et al. 2009, 2012). In addition, Peck et al. (2016) showed that

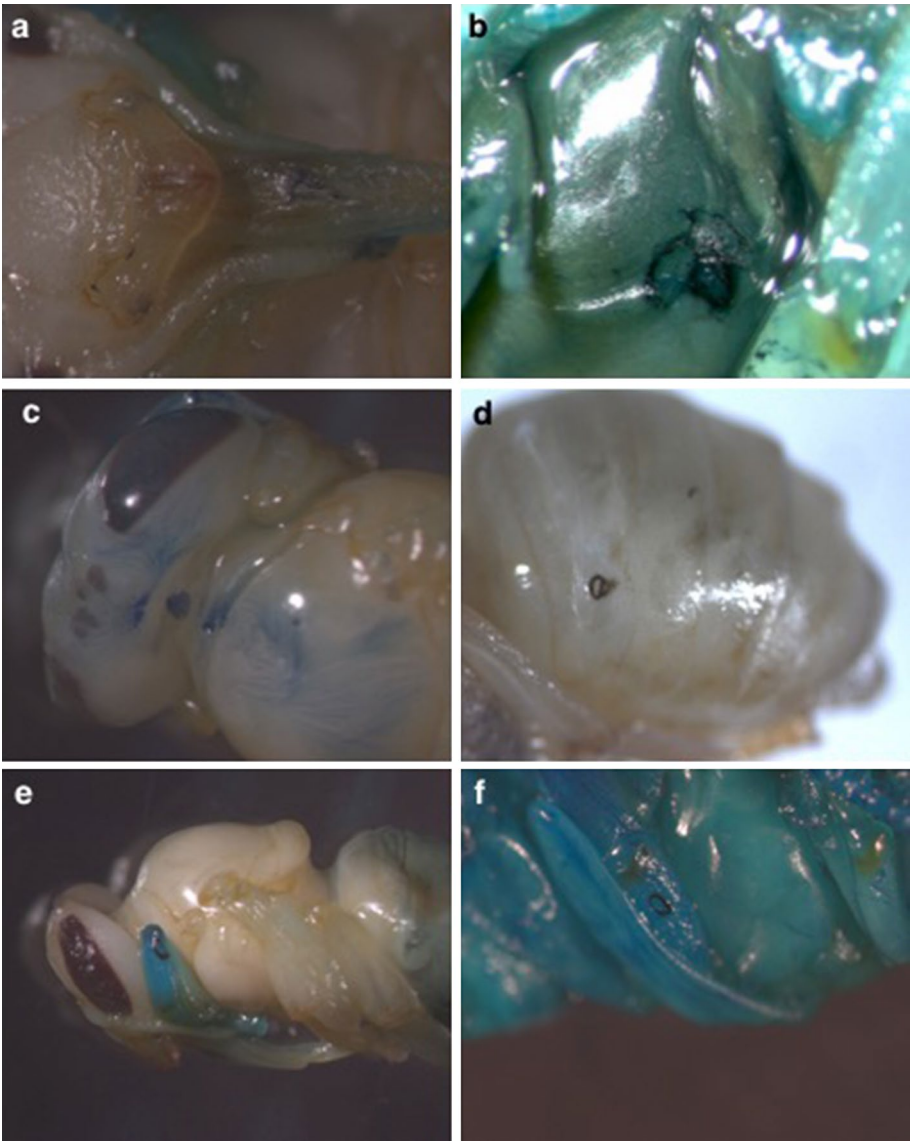


**Fig. 6** Vital staining of honey bee pupa after infestation by *female Varroa destructor*. **a** Head, **b** thorax, **c** between head and thorax, **d** abdomen, **e,f** leg

mites had the strongest preference for the thorax and abdominal segments on adult foraging bees.

Information of feeding site preferences may offer some insight into varroa nutritional needs, used for developing artificial diets. Studies of varroa mites suggest that varroa females feed exclusively on hemolymph (Cabrera et al. 2017; Erban et al. 2015). Hemolymph surrounds every cell in the hemocoel of most arthropods (Fredrick and Ravichandran 2012; Lawry 2006; Paska et al. 2014), and is also a reservoir of nutrients. The

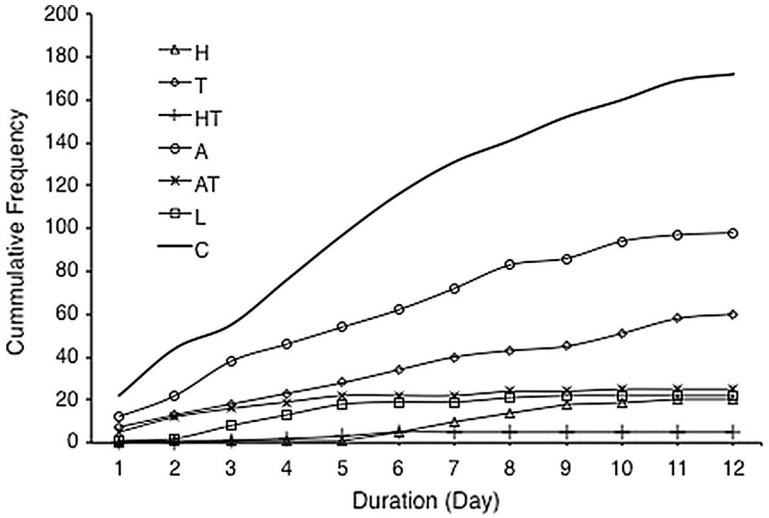


**Fig. 7** Cumulative frequency of mites found on various body regions: head (H), thorax (T), between head and thorax (HT), abdomen (A), between abdomen and thorax (AT), and leg (L), or on the capsule wall (C)

abdomen likely contains a high volume of hemolymph. Additionally, the abdomen contains the bulk of the honey bee's fat body, which is a site of nutrient synthesis and storage, including carbohydrate, protein, lipid macronutrients and important micronutrients. The insect peripheral fat body is located just beneath the cuticle and extends over most of the body (Chapman 1998). Previous studies have shown a high level of protein accumulation in the form of vitellogenin as a major constituent of the honeybee fat body (Amdam et al. 2003; Arrese and Soulages 2010; Nilsen et al. 2011). Fat body cells from honeybee hosts infested with varroa mites were reduced in size, and depleted of sequestered substrates, possibly vitellogenin (Drescher and Schneider 1987), suggesting varroa may be directly targeting fat body constituents as food.

In our study, mites successfully fed from body parts other than the abdomen (see Fig. 6). Interestingly, varroa females were also observed for periods on the eyes of the honey bee pupa during these experiments. Thus, we can speculate that a food source is present on every part of the host's body, but perhaps more readily available in the abdomen because of its size and large surface area and volume. Further studies are needed to clarify the dietary needs of female varroa mites; the specific nutrients sequestered from the host, and the metabolic processes critical for their reproduction, are crucial data for developing a fully artificial in vitro rearing system for these mites.

## Additional observations

### Defecation activity

We recorded daily mite excretory behavior as evidenced by traces of whitish fecal matter on the wall of capsules and on hosts (Fig. 5c). Previous research has suggested varroa females establish preferred defecation sites mostly on the posterior end of the cells

(Calderon et al. 2009, 2012). We observed fecal deposits at multiple sites on the gel capsule walls, suggesting that varroa may not necessarily have a strong preference for a defecation site (Fig. 5c). However, this behavior may be related to the larger volume of the gel capsule (9.0 mm wide, 15 mm deep), allowing mites more space compared to the natural brood cell (4.9 mm wide, 12 mm deep). Nonetheless, even provided more space, varroa females defecated on host integument, despite suggestions that this behavior may reduce their own fecundity (Calderon et al. 2012; Donze and Guerin 1994).

## Mite reproduction

Although not a focus of our studies, we observed reproduction of 7.7% (n = 52) females from trial 1. Figure 8a shows a representative outcome of varroa reproduction: varroa female and two of her offspring, 1 male and 1 female deutonymph. The honeybee pupa is obviously shrunk in size due to loss of water possibly from wounds. Figure 8b shows a varroa nymph from another female. Reproduction in mites has been recorded in other studies (de Guzman et al. 2013; Nazzi et al. 2002). These studies and previous pioneering studies (Chiesa et al. 1989; Donze et al. 1998; Nazzi and Milani 1994) have achieved some success in getting mites to reproduce. While these studies seem satisfactory, the success recorded may be connected with the use of foundress females (collected from already-wax capped host brood cells) at the peak of their reproductive readiness for in vitro experiments, or even using foundresses in their natural cells for studies. However, these experiments do not report that first generation progeny from these experiments was able to produce a second generation of mites.

Low instances of successful reproduction of phoretic-stage female mites reported here might provide insights into the cues and/or physiological status required for varroa reproduction. For example, vital chemical cues (kairomones and pheromones) specific to precise host stage or the host's natural environment might have been omitted from our experiments (Frey et al. 2013; Heinz and Nikolaus 1986; Kirrane et al. 2011). Females used in our experiment were harvested as phoretics, and although mated, there was little or no additional information about the mite's physiological condition. Spending some time in the phoretic stage may be necessary for female mites to prepare for oocyte development

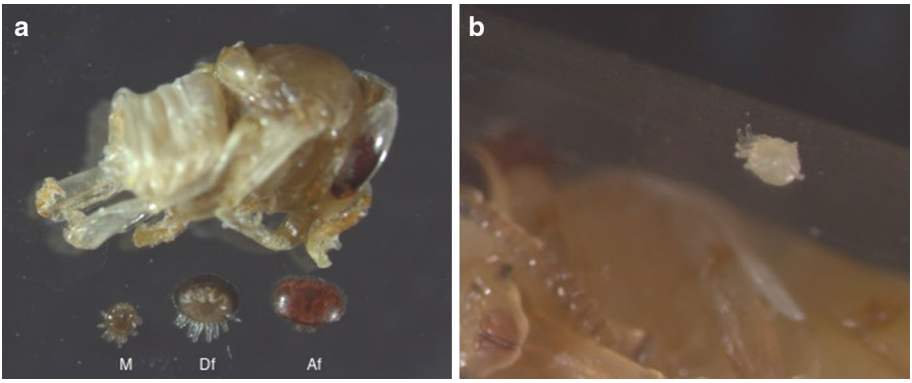


**Fig. 8** *Varroa destructor* reproduction: **a** son (adult male, M), deutonymph (Df), and mother (adult female, Af) mite after artificial infestation on honey bee pupa. **b** A nymph on the capsule wall indicating survival and molting of the F1 generation

and subsequent oviposition. The factors providing for successful reproduction in observed cases cannot be determined here. Nonetheless, our findings raise questions of what fundamental role the phoretic stage plays in the varroa life cycle, other than transportation to another brood cell to initiate the next cycle of reproduction. Our findings also suggest the importance of selecting either phoretic or foundress stage female mites, when designing experiments of varroa reproduction, longevity, or other aspects of their biology, and parasite–host interactions.

## Conclusion

Longevity studies of *V. destructor* mites on their honey bee host have been conducted using in vitro techniques. These studies demonstrate that it is possible to maintain female varroa mites in a semi-artificial laboratory colony with long-term survival and (limited) reproduction. The VMS system described herein shows that it is possible to maintain female varroa mites under in vitro conditions for many days. Adequate conditions for varroa mites and honey bee pupae were 32.5 °C and 85% RH. Replacing the pupal hosts every 6 days with young pupae enhanced varroa mite survival. The VMS system may be useful for testing miticides or other mite control methods, as it can be done under conditions that simulate the physical conditions in the natural host environment.

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