College of Agricultural and Life Sciences
Department of Entomology and Nematology

Charles Steinmetz Hall
Bldg. 970, Natural Area Dr
PO Box 110620
Gainesville, FL 32611-0620
352-273-3901
352-392-0190 Fax

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Development of *Varroa destructor in vitro* rearing methods

Cameron Jack[1], Jamie Ellis[2]

[1]Co-PI: Department of Entomology and Nematology, University of Florida, Steinmetz Hall, Natural Area Dr., P.O. Box 110620, Gainesville, FL 32611-0620. cjack@ufl.edu

[2]PI: Department of Entomology and Nematology, University of Florida, Steinmetz Hall, Natural Area Dr., P.O. Box 110620, Gainesville, FL 32611-0620. jdellis@ufl.edu

1. Introduction

The number of honey bee colony losses described in recent reports is staggering (Lee et al., 2015; van der Zee et al., 2015). While reasons for this decline are multifaceted, the parasite *Varroa destructor* is considered by many honey bee researchers as the greatest contributing factor to honey bee colony losses (Rosenkranz et al., 2010; Dietemann et al., 2012). With a nearly global distribution (Iwasaki et al., 2015), *Varroa* will severely weaken or cause the collapse of most honey bee colonies if left untreated (Thompson et al., 2014; Frey & Rosenkranz, 2014). Despite collaborative efforts from insect pathologists, acarologists, and apiculturists, scientists have yet to produce long-term solutions for controlling *Varroa*.

As *Varroa* continue to decimate honey bee populations worldwide, the development of new and innovative mite control methods should remain a priority among honey bee researchers. Currently, research efforts aimed at controlling *Varroa* focus on effective acaricides, mechanical controls, biological controls, RNA interference, and breeding tolerant lines of bees. One necessary element in all of these experiments is the need for *Varroa*. Presently, there is no other way to obtain *Varroa* for laboratory experiments other than finding infected colonies and removing the *Varroa* from adult bees or capped brood cells (Dietemann et al., 2013).

The current process of obtaining *Varroa* for experimentation is dependent upon season and weather conditions, is time consuming, and can be costly (Dietemann et al., 2012). Additionally, in order to obtain the number of mites necessary for experimentation, researchers have to leave their colonies untreated, which can greatly affect their survival and pose a risk to other colonies nearby. The development of an *in vitro Varroa* rearing technique would rapidly advance our ability to screen potential acaricides, breed bees for *Varroa* tolerance, understand *Varroa*

biology, and more. Such a technique also would allow for year-round *Varroa* research and increase standardization of experiments leading to greater reproducibility.

Development of an *in vitro* rearing method is quite challenging as *Varroa* has very specific lifecycle requirements (Rosenkranz et al., 2010). Nearly three decades ago, several attempts to create an *in vitro* rearing technique were made, but virtually all attempts failed to yield reproductively mature second-generation mites (Abbas and Engels, 1988; Bruce et al., 1988; Chiesa et al., 1989). Nazzi and Milani's (1994) approach used gelatin capsules containing freshly capped larvae and captured mites. Bruce et al. (1991) created an artificial diet using ground honey bee larvae in an attempt to feed *Varroa* through a membrane. Both experiments showed some promise and should be retested to determine if they warrant continued attention.

Although previous efforts to develop an *in vitro* rearing technique for *Varroa* have been unsuccessful, recent advances provide us with greater opportunities for success. One particular advancement is the development of *in vitro* honey bee rearing technique. *In vitro* honey bee rearing techniques have been refined in order to test the effects of different chemicals on the development of honey bee larvae (Schmehl et al. 2016). Attempting to rear *Varroa* on honey bee larvae reared *in vitro* has never been tried before; nevertheless, this technique may provide a more natural environment for the reproduction of the mite.

The aim of our study is to provide a necessary foundation for the development of a successful *in vitro Varroa* rearing protocol. We created two separate experiments to achieve this objective. For the first experiment, we determined which *Varroa* rearing method yields the greatest number of offspring. The purpose of the second experiment was to refine the most successful method in order to improve *Varroa* rearing rates.

2. Materials and Methods

2.1 Experiment 1

Recently capped brood from established colonies of European-derived honey bees (*Apis mellifera ssp.*) at the University of Florida Honey Bee Research and Extension Laboratory (Department of Entomology and Nematology, Gainesville, FL) was brought to the laboratory and kept in an incubator at 34.5° C and 65% relative humidity (RH). Shortly thereafter, the capped prepupae and pupae were removed from the cells individually and *Varroa* females that had not yet begun to lay eggs were collected for experimentation. This collection procedure ensured that reproductive *Varroa* females were used throughout the experiment for each rearing method. Pilot studies testing several *Varroa* rearing methods were performed in the spring of 2016. These methods included the use of gelatin capsules, group rearing, rearing on honey bee larvae reared *in vitro*, and rearing on an artificial diet.

2.1.1 Gelatin Capsules Method

Worker bee prepupae were collected following the methods of Chiesa et al. 1989 in order not to damage the fragile prepupae. Briefly, combs containing capped brood were collected and placed into the incubator at 34.5° C and 65% RH for about 2-3 hours. Paper was placed underneath the brood combs to catch prepupae that would crawl out of their cells, a normal behavior of

prepupae whose cells have yet to be capped by worker bees. The prepupae were collected from the paper or directly off of the combs and placed into size "0" gelatin capsules (Now Foods, Bloomingdale, IL, USA) 7 mm in diameter. Small ventilation holes were added in the capsules using a number 2 pin. *Varroa* females were inserted into the gelatin capsules with the larvae and capsules were placed vertically into an empty micro-pipette tip container (Fig 1).

2.1.2 *Group Rearing Method*

Worker bee prepupae and *Varroa* females were collected as described previously. Ten *Varroa* and ten prepupae were placed into a sterile Falcon® 50 × 9 mm petri dish (Corning Inc., Corning, NY, USA) (Fig 2).

2.1.3 in vitro Reared Larvae Method

Honey bee larvae were reared *in vitro* following the methods described in Schmehl et al. (2016). Briefly, 1st instar worker larvae were grafted from brood combs to sterile plastic 48-well plates. These larvae were fed prescribed larval diets and transferred to new sterile plates once ready to pupate (Fig 3). A cork plug containing several ventilation holes was placed on each cell to ensure the *Varroa* would remain in the designated cell (Fig 4).

2.1.4 Artificial Diet Method

Bruce et al. (1988) found relative success by creating small *Varroa* rearing chambers using modified honey bee queen rearing cells (Fig 5). We followed this method and placed one or two *Varroa* females in each chamber (Fig. 6). Parafilm was stretched over the top of the chambers to create a membrane that the mites were able to pierce in order to feed on the artificial diet placed upon the Parafilm. Hemolymph was extracted and fat tissue was dissected from live 5th instar honey bee worker larvae and placed directly on the Parafilm membrane. The hemolymph and fat tissue were replaced twice daily, but desiccation was a frequent problem. The artificial diet method was quickly eliminated from the study as mite feeding and survival was much lower using this protocol than when using others.

The remaining methods were tested at 34.5°C and either 35% or 75% RH. The lower humidity offered less contamination from mold while the higher humidity is closer to natural in-hive conditions. Each method was repeated until at least 100 mites survived for an 11-day period. The survival of the bees, the mites and any progeny produced by the mites was recorded. For this experiment, only instances where both the mites and the bees survived for the duration of the 11-day period were counted.

2.2 Experiment 2

Environmental conditions such as humidity and cell size were manipulated in an attempt to improve *Varroa* reproduction. A 3 × 2 full factorial design was used to test all possible combinations of gelatin capsule size (size "0" or "2", 7 mm and 6 mm diameter, respectively) as well as relative humidity (65, 75, or 85%). One hundred capsules each containing one worker bee prepupae and one *Varroa* female were allocated to each treatment for an 11-day period. The survival of the bee, the mite and any progeny produced by the mite were recorded for comparison.

The reproductive success of the F1 mites was also observed during Experiment 2. Fully developed, sclerotized female daughters of the original foundress mites were collected for additional testing. These mites were placed into a new gelatin capsule with a fresh worker honey bee prepupae under the environmental conditions as described previously. These F1 mites were observed after an 11-day period.

2.3 Statistical Analysis

Logistic regression models were created for survival and reproduction data in both experiments. Experiment 1 data were analyzed using a one-way ANOVA, recognizing the rearing method (gelatin capsule, *in vitro* honey bee, and group rearing methods) and relative humidities (35 and 75%) as the main effects. Each combination of rearing method and humidity level was recognized as a separate treatment. There was no reproduction among the group reared mites; therefore, this method was removed from further statistical analyses. Experiment 2 data also were analyzed using a one-way ANOVA, recognizing gelatin capsule size ("0" or "2") and relative humidity (65, 75, and 85%) as the main effects. Each combination of capsule size and humidity level was recognized as a separate treatment. The treatment means in both experiments were compared using Tukey's Honest Significant Difference test using $P \leq 0.05$. All statistical analyses were conducted using R software (R-Core-Team, 2015).

3. Results

3.1 Experiment 1

Survivability varied between treatment groups ($P<0.0001$). *Varroa* reared using the *in vitro* reared larvae method at 75% RH had significantly higher survival rates (80.5%) than did those reared all other ways. This was followed by *Varroa* reared in gelatin capsules at 75% RH, which had significantly higher survival rates (64.2%) than those of the *Varroa* reared the remaining ways (Fig 7). The group rearing method at 75% RH yielded the lowest *Varroa* survival rate (9.4%) of any treatment.

Varroa reproductive success varied between each treatment group ($P<0.0001$). Both the RH and the rearing method significantly affected the number of *Varroa* female offspring ($P<0.0001$). The number of reproducing females was significantly higher in the gelatin cells at 75% RH (68) than that for any other treatment (Fig 8). The number of reproducing *Varroa* females in the *in vitro* reared larvae 75% RH (39) treatment was significantly higher than that reared via either method at 35% RH. There were large differences in the number of *Varroa* reared via the gelatin capsule method (22 females and 63 males) and the *in vitro* reared larvae method (2 females and 3 males) (Table 1).

3.2 Experiment 2

Survivability again varied between treatment groups ($P<0.0001$) with both humidity ($P<0.0001$) and capsule size ($P=0.029$) significantly impacting *Varroa* survival. *Varroa* reared in the 7 mm diameter capsules at 65 and 75% RH had significantly higher survival rates (33 and 32%, respectively) than did those of the other treatments (Fig 9).

Varroa reproductive success did not vary significantly between treatment groups (*P>0.05*). There were only 12 F1 mites in the 7 mm capsules at 75% RH. Of these, 11 survived the next 11-day period, though none were observed to reproduce. We observed the largest number of mature *Varroa* female offspring and mature *Varroa* male offspring in the 7 mm capsules at 75% RH (Table 2).

4. Discussion

Our study provides an important foundation for the development of future *in vitro Varroa* rearing protocols. According to our knowledge, this is the first study to compare several methods used in previous attempts to rear *Varroa in vitro* and include the untested method of rearing *Varroa* on *in vitro* reared honey bee larvae. We found that the gelatin capsule method of *in vitro Varroa* rearing yielded high *Varroa* survival while also facilitating the most mite reproduction, especially fully mature males and females (Experiment 1). Further, our results suggest that *Varroa* reared in the 7 mm diameter gelatin capsules have higher survival rates than those reared in smaller ones (Experiment 2).

We reared *Varroa* on worker honey bee pupae, even though mites are significantly more successful at reproducing in drone honey bee cells (reviewed by Rosenkranz et al., 2010). Worker honey bees were selected for this study as drones are only reared at certain times of the year and can prove difficult for some researchers to obtain when necessary. Ultimately, the most ideal *Varroa* rearing method would facilitate mites feeding on a substance other than on living honey bees. If achieved, *Varroa* could be reared year round.

Designing rearing protocols is a very difficult task, as there are many reasons a method might work for some, but not for others. *Varroa* survival was low in the group rearing method (Fig 7) and none of the mites reared this way reproduced. A possible explanation for this may be that *Varroa* and the bee pupae need a more confined space than that available in the petri dishes. The hemolymph and fat tissue dried out in just a few hours, preventing the mites inside the chambers described in section 2.1.4 from feeding well. While the mites and *in vitro* reared bees had reasonable survival rates, the mites had much lower reproductive success (Fig 8).

Rearing *Varroa* in gelatin capsules appears to be the most likely method to achieve long term success moving forward, as both the survival and reproductive rates were high for *Varroa* in this method. Fortunately, this method is inexpensive, can be prepared quickly, and requires less technical skill than any of the other methods tested. Additionally, rearing *Varroa* in gelatin capsules does not require any specialized or expensive equipment aside from an incubator. Gelatin capsules are also clear, allowing non-intrusive observations and opportunities to photograph mite reproduction. Future studies of *Varroa* behavior could be conducted inside gelatin capsules (Nazzi and Milani, 1994).

Several observations should be noted in order to improve the success rate of *Varroa* rearing in gelatin capsules. For instance, mold can become an issue inside the incubators, especially during the first three days of rearing *Varroa*, as some prepupae die after transfer to the capsules. Thus, the capsules should be checked and those with dead prepupae should be removed at least every

24 hours, as mold contamination could ruin a group of reproducing mites. At 85% RH, mold becomes a significant problem and causes the gel to become too soft. If the gel becomes too soft, the top and bottom pieces of capsule will fuse together, making it necessary to cut open the capsule if one wants to collect the mites inside. The soft gel also reseals the ventilation holes, increasing the likelihood of bee mortality and pupae sticking to the sides of the soft capsules.

The most ideal situation for *Varroa* reproduction appears to be inserting mites into 7 mm gelatin capsules at 75% RH. This is fortunate, as 7 mm gelatin capsules are large enough that very few prepupae are damaged when transferred into the capsules, unlike for the 6 mm gel capsules. Gelatin capsules 7 mm in diameter are also large enough to accommodate drone prepupae if desired. Additionally, 75% RH can be obtained by most humidity controlling incubators, allowing more people to use this method of *in vitro Varroa* rearing.

A recent study conducted at the University of Maryland suggests that *Varroa destructor* may not feed entirely on honey bee hemolymph as is presently believed (pers. comm., S. Ramsey), but on honey bee fat tissue. If *Varroa* do feed on honey bee fat tissue, this could dramatically improve *in vitro* rearing methods.

An *in vitro Varroa* rearing protocol has the potential to improve certain aspects of *Varroa* research significantly. If in the future we are successful in developing such a tool, *Varroa* could be studied year round with increased accuracy and efficiency. Future work in this field must not be abandoned, as the impact of *Varroa* on honey bee health remains devastating and the potential benefit of an *in vitro Varroa* rearing protocol is incalculable.

5. References

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Table 1. Number of *Varroa* progeny produced via the most effective *in vitro* rearing techniques.

Method	Protonymphs	Deutonymphs	Mature Males	Adult Females
gelatin capsules	71	17	63	22
in vitro honey bee	76	12	3	2

Table 2. Number of *Varroa* progeny produced in Experiment 2.

Treatment	Protonymphs	Deutonymphs	Mature Males	Adult Females
6 mm 65% RH	17	3	0	0
7 mm 65% RH	11	3	2	0
6 mm 75% RH	13	0	1	0
7 mm 75% RH	10	0	12	13
6 mm 85% RH	15	1	1	0
7 mm 85% RH	11	0	0	0

Figure 1. Honey bee prepupae and *Varroa* females placed inside 7 mm diameter gelatin capsules. The capsules are placed vertically in an empty micro-pipette tip box.

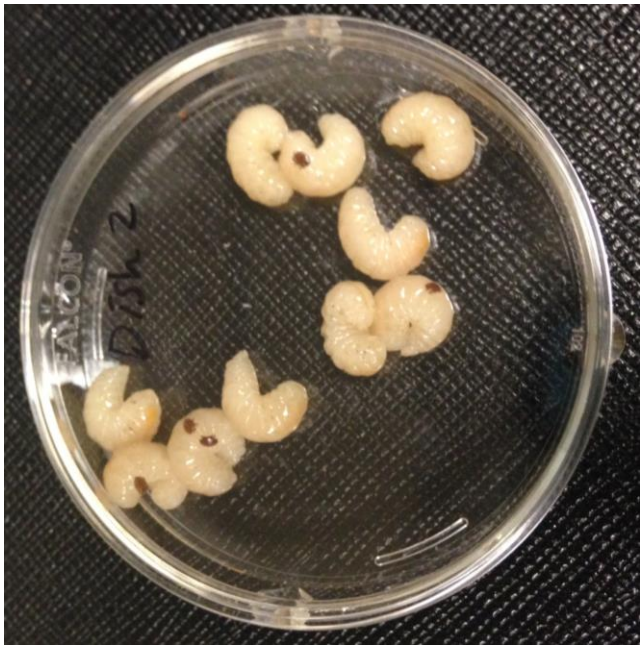

Figure 2. Ten mites were placed in a sterile Falcon® 50 × 9 mm style petri dish along with ten worker bee prepupae. In this method, the mites are able to feed on any of the honey bee prepupae.

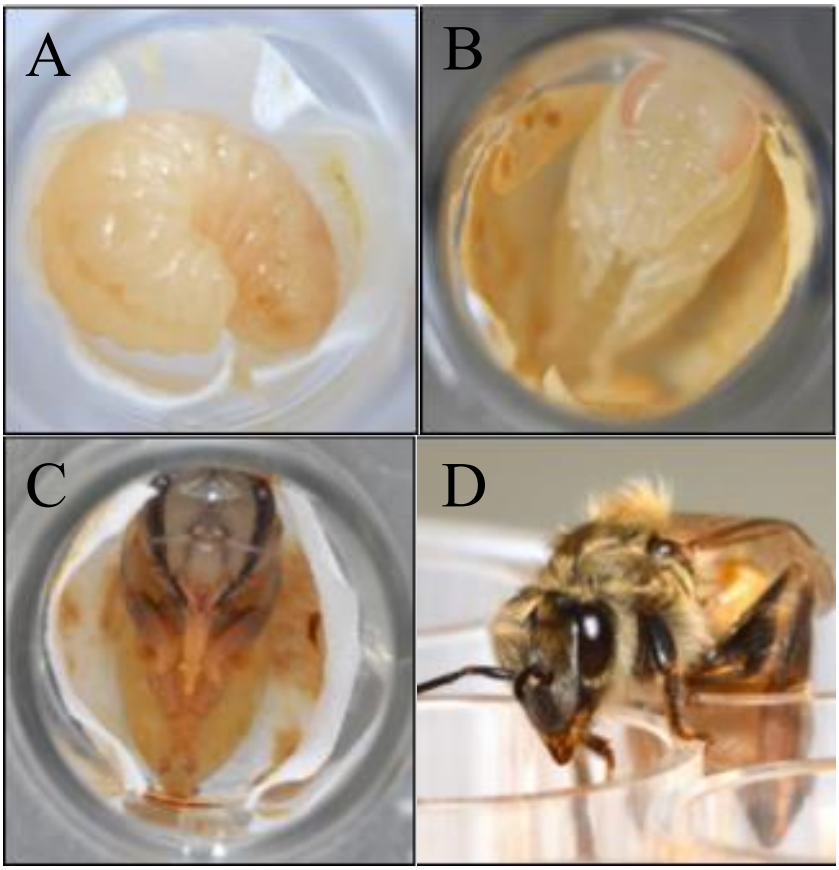

Figure 3. Development of a honey bee reared *in vitro*. A) Prepupa is transferred to a new sterile plate. B) Pupa about 5 days after transfer. C) Pupa about 9 days after transfer. D) Newly emerged adult about 12 days after transfer.

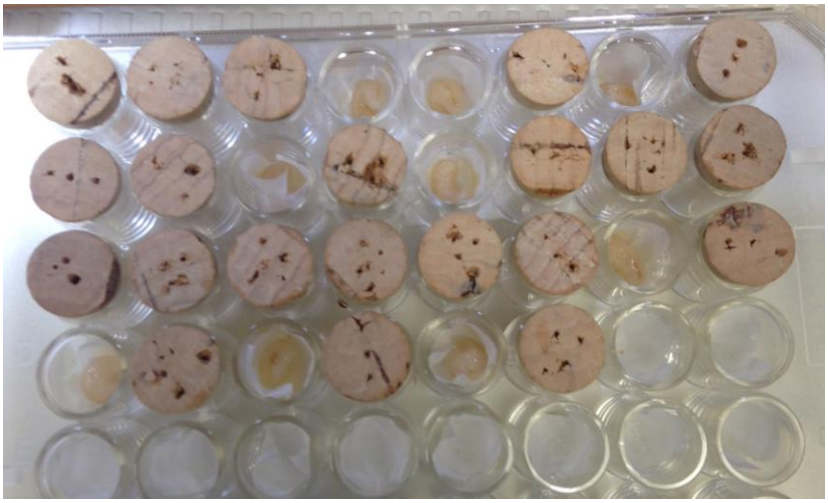

Figure 4. *Varroa* are placed in wells containing *in vitro* reared honey bee prepupae. The wells are closed with cork plugs.

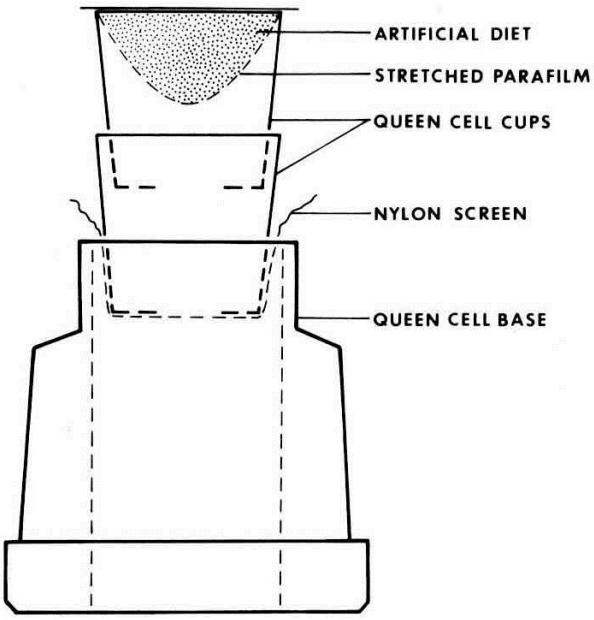

Figure 5. Modified honey bee queen rearing cell to feed and rear *Varroa*. Figure is from Bruce et al. (1988).

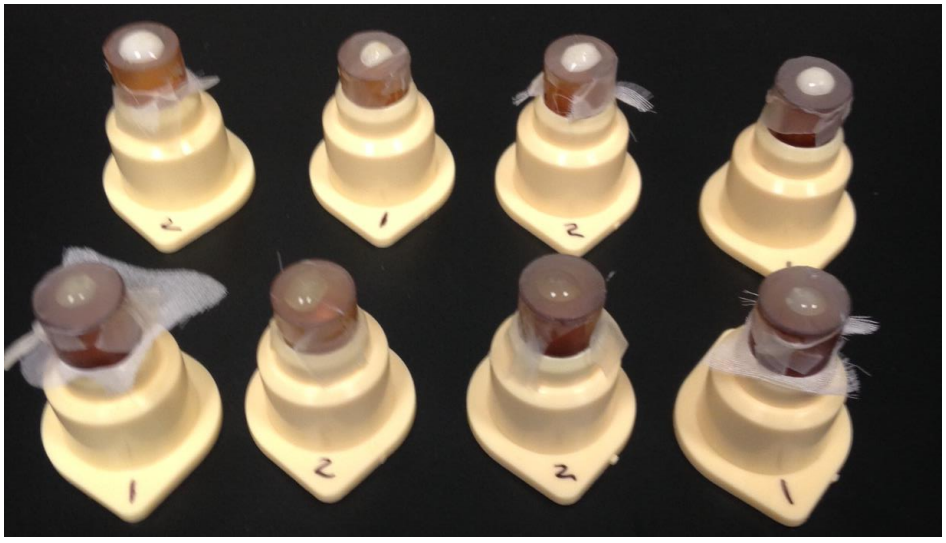

Figure 6. Chambers created based on Bruce et al. (1988). They are designed to test feasibility of feeding honey bee hemolymph or fat tissue to *Varroa*. The chambers in the top row contain fat tissue from dissected honey bee prepupae placed on the Parafilm membrane. Those on the bottom row contain honey bee prepupae hemolymph placed on the membrane. The numbers on the chambers indicate how many mites are inside each chamber.

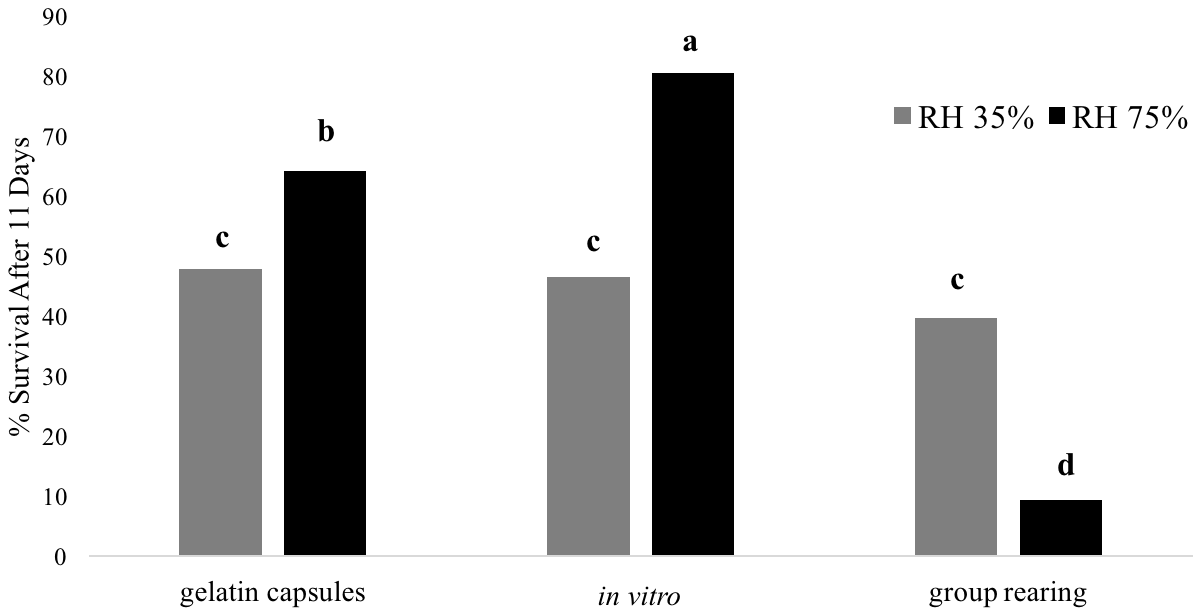

Figure 7. Comparison of the percent of surviving individuals (bees and mites both must survive to count as "survived") at two different humidities across three *in vitro Varroa* rearing methods. Significant differences at $P \leq 0.05$ among groups are indicated by different letters.

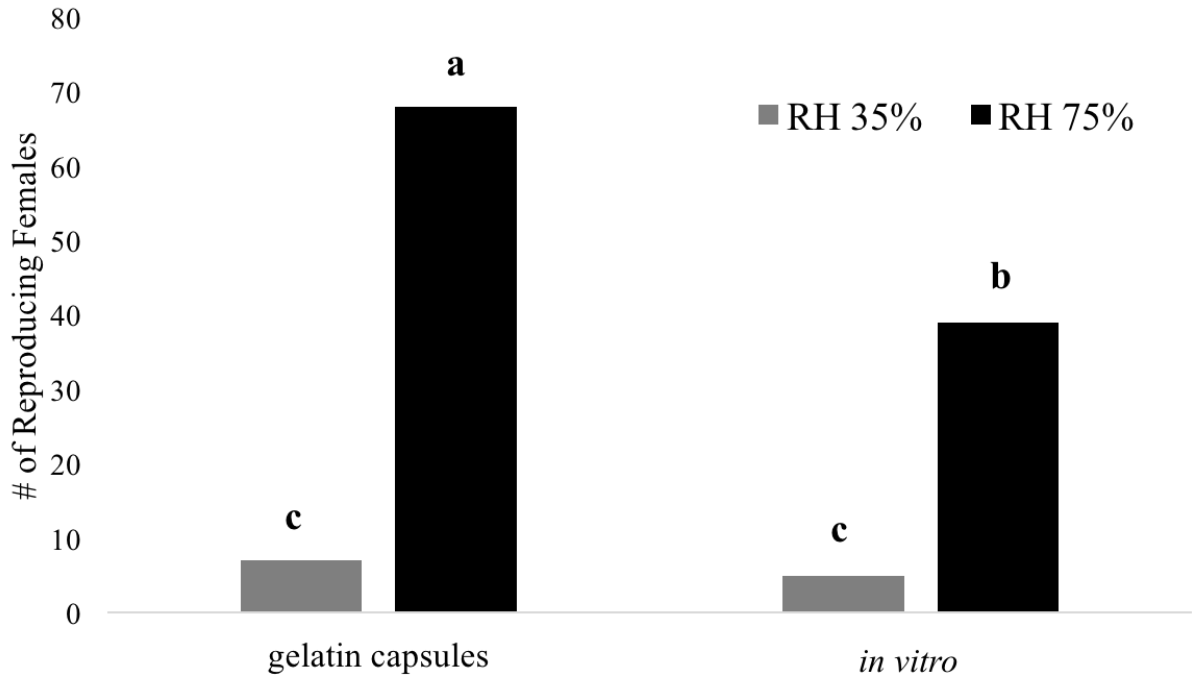

Figure 8. Comparison of the number of reproducing female mites at two different humidities using the gelatin capsule method and *in vitro* reared honey bee method. Significant differences at $P \leq 0.05$ among groups are indicated by different letters.

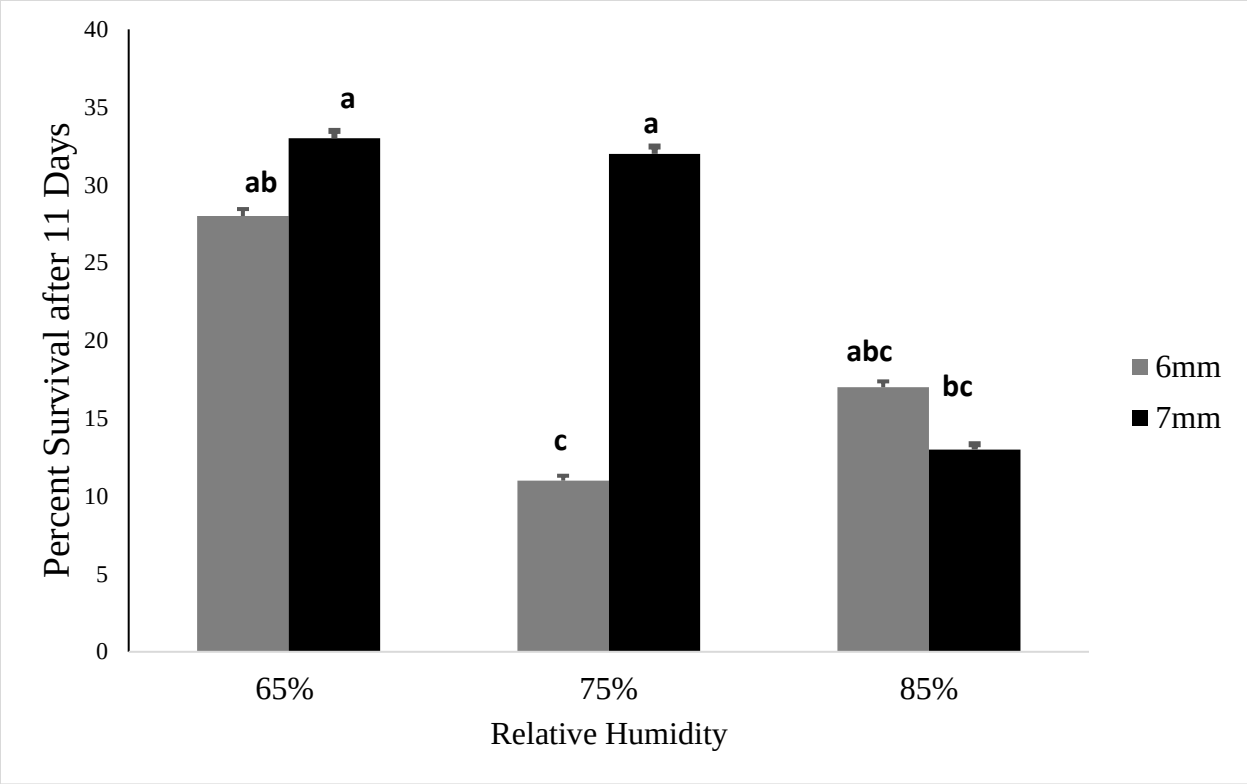

Figure 9. Comparison of the percent of surviving *Varroa* (bees and mites both must survive to count as "survived") at two different gelatin capsule sizes and across three levels of humidity. Significant differences at $P \leq 0.05$ among groups are indicated by different letters.