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A comparison of *Varroa destructor* (Acari: Varroidae) collection methods and survivability in in vitro rearing systems

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Abstract

Varroa destructor Anderson & Trueman (Parasitiformes: Varroidae) is an ectoparasitic pest of the western honey bee (*Apis mellifera* L.; Hymenoptera: Apidae) colonies. The ability to study all life stages of the mite in a laboratory setting requires one to rear the mite in vitro. This is a crucial step for the advancement in research studies, and the development of management protocols for *Varroa*. Current practices require that *Varroa* be collected from field colonies for use in lab-based studies. Traditional collection techniques for obtaining mites from adult bees include using carbon dioxide or a method in which a combination of powdered sugar and shaking dislodges the mites from a group of adult bees (i.e., a "sugar shake"). Herein, we compared 2 mite collection techniques and measured mortality of the mites after collection using the *Varroa* maintenance system, a tool for maintaining in vitro populations of *Varroa* on their host. Our results indicate that mites collected using the sugar shake method lived significantly longer (> 6 d, with 20% mortality at 6 d) than did those collected using carbon dioxide (3.9 d, with 66% mortality at 6 d). Carbon dioxide exposure was detrimental to the recovery of *Varroa*. These data provide critical information on how to collect *Varroa* properly for use in in vitro survival studies.

Key Words: honey bees; *Varroa destructor*; carbon dioxide; sugar shake

Resumen

Varroa destructor Anderson & Trueman (Parasitiformes: Varroidae) es una plaga ectoparásita de las colonias de abejas melíferas occidentales (*Apis mellifera* L.; Hymenoptera: Apidae). La capacidad de estudiar todos los estadios de la vida del ácaro en un entorno de laboratorio requiere la cria del ácaro in vitro. Este es un paso crucial para el avance de los estudios de investigación y el desarrollo de protocolos de manejo para *Varroa*. Las prácticas actuales requieren que se recolecte *Varroa* de colonias de campo para su uso en estudios de laboratorio. Las técnicas tradicionales de recolección para obtener ácaros de abejas adultas incluyen el uso de dióxido de carbono o un método en el que una combinación de azúcar en polvo y agitación desprende los ácaros de un grupo de abejas adultas (un "batido de azúcar"). Aquí, comparamos 2 técnicas de recolección de ácaros y medimos la mortalidad de los ácaros después de la recolección utilizando el sistema de mantenimiento de *Varroa*, una herramienta para mantener poblaciones in vitro de *Varroa* en su hospedero. Nuestros resultados indican que los ácaros recolectados usando el método de batido de azúcar vivieron significativamente más tiempo (> 6 dias, con el 20% de mortalidad a los 6 dias) que los recolectados usando dióxido de carbono (3.9 dias, con el 66% de mortalidad a los 6 dias). La exposición al dióxido de carbono fue perjudicial para la recuperación de *Varroa*. Estos datos proporcionan información crítica sobre cómo recolectar *Varroa* correctamente para su uso en estudios de sobrervivencia in vitro.

Palabras Clave: abejas melíferas; *Varroa destructor*; dióxido de carbono; batido de azúcar

Varroa destructor Anderson & Trueman (Parasitiformes: Varroidae) is an obligate ectoparasite of the western honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) (Nazzi et al. 2016). This mite is a significant factor in the decline of populations of managed honey bee colonies in many parts of the world (Fazier et al. 2010; Nazzi et al. 2016). The mite damages bees by feeding on bee fat bodies (Ramsey et al. 2019) and transmitting pathogens to parasitized bees (Forfert et al. 2015).

The ability to rear *Varroa* in vitro is a crucial step for scientific study and for developing management protocols for this pest (Dietemann et al. 2013). To rear *Varroa* in vitro, the mite must first be collected from honey bee colonies to start the initial rearing population. The collection method used will depend on the adult life cycle phase and the goal of the experiment. A female *Varroa* has 2 stages in its life cycle, i.e., reproductive and non-reproductive. A non-reproductive female mite attaches itself to an adult honey bee on which it feeds. When honey bee brood is available, the non-reproductive stage lasts about 5 to 11 d after which the mite leaves the adult bee, invades a brood cell about to be capped, and begins to feed and reproduce on the immature honey bee contained within the cell (Rosenkranz et al. 2010; Häußermann et al. 2016). If there is no brood available for reproduction, female *Varroa* may remain non-reproductive for over 6 mo; this is usually the case in colder climates where mites will overwinter on adult bees (Rosenkranz et al. 2010; Huang 2012).

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Various collection methods exist for harvesting reproductive and non-reproductive mites intended for in vitro studies. Reproductive female mites may be collected manually by opening brood cells, removing the cell contents, and selecting the mature *Varroa* (Human et al. 2013; Egekwu et al. 2018). Reproductive female mites may also be collected from L5 larvae in recently capped brood cells (Abbas & Engels 1988; Chiesa & Milani 1988; Chiesa et al. 1989). Non-reproductive female mites may be collected from bees within the hive by washing bees with water or alcohol, using inert dusts, or using carbon dioxide (Macedo et al. 2002; Dietemann et al. 2013; Bahreini & Currie 2015). Only 2 methods, inert dusts (i.e., powdered sugar) and carbon dioxide, may be well suited for collecting live mites for in vitro studies. However, there are no data comparing the 2 methods of collection.

Furthermore, there exists no investigation examining the effects of these 2 methods on *Varroa* maintained in vitro. The carbon dioxide method, whereby cohorts of bees are gassed with carbon dioxide and the adult mites that fall from them are collected, is used widely (Büchler 2015; Oliver 2017; Rosenkranz unpublished data), but mites collected this way seem to have high mortality rates (personal observation). For the powdered sugar method, a cohort of adult bees is dusted with sugar, allowed to tumble in the sugar for a set time (usually about 2 min), and then shaken to dislodge and collect the mites (Dietemann et al. 2013). Given that these 2 methods are available to collect *Varroa*, we developed a study whereby we could determine the impact of both collection methods on mite mortality in vitro. Our data provide critical information for those who want to conduct future research on *Varroa* in vitro.

Materials and Methods

VARROA COLLECTION

Source of *Varroa*

Varroa were collected from 3 honey bee hives maintained at the USDA-ARS, Center for Medical, Agricultural, and Veterinary Entomology (USDA-ARS, CMAVE), Gainesville, Florida, USA. Colonies were managed to have high mite populations, i.e., they were not treated with chemicals to reduce mite infestations. The bee colonies were kept in standard Langstroth hives (Dadant & Sons, Inc., High Springs, Florida, USA) containing 10 brood frames and were placed on wooden platforms approximately 54 cm above the ground. Both harvest methods were applied to each colony, and the bees were returned to the colonies after the collections were completed.

Mite Collection by Carbon Dioxide Method

A stainless-steel collection device was constructed to facilitate the collection of mites using carbon dioxide (Fig. 1). Two 300 mL stainless steel canisters measuring 65 mm in diam and 95 mm in height (WebstaurantStore, 407DR10T, Lititz, Pennsylvania, USA) were used to construct the device. The canister that contained the bees had a 6.35 mm brass bulkhead union (Swagelok, B-400-61, Jacksonville, Florida, USA) inserted through the side that served as an entry port for the introduction of carbon dioxide. A 7 cm diam piece of aluminum wire mesh (3 openings per cm; Phifer Vent Mesh, Tuscaloosa, Alabama, USA) was sandwiched between 2 metal jar lid bands (Ball® lid band, 1033976; Walmart, Gainesville, Florida, USA) and soldered together allowing the 2 canisters to be attached. For the collection of non-reproductive mites, approximately 50 to 100 bees were gently brushed off frames into the 300 mL stainless steel canister. The 2 canisters were then screwed together using the modified lid. Carbon dioxide gas was introduced into the collection device using a dust-removing gun (AMR-CO-2016; American Recorder Technologies, Simi Valley, California, USA) containing a threaded 16 g carbon dioxide cartridge (CO-42104; American Recorder Technologies, Simi Valley, California, USA). The amount of carbon dioxide introduced into the canister was enough to only immobilize the bees (1 s pull of the trigger). Once induced syncope was achieved, the canister was inverted and lightly shaken for about 30 s. The mesh held the bees in place while the mites passed through into the adjoining canister. The mites were collected and placed in a Petri dish containing a moistened paper towel. The jars were disconnected and the bees were introduced back into their hive.

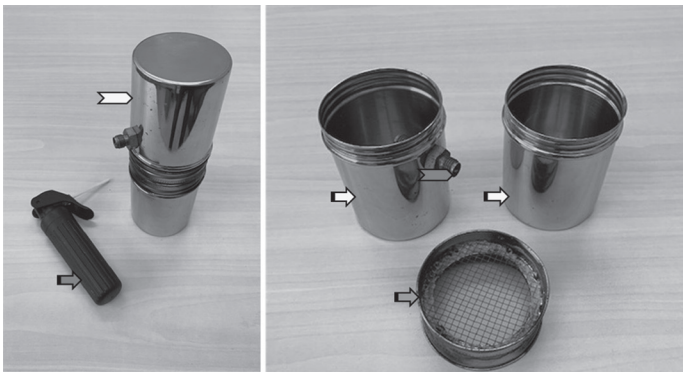

Fig. 1. The stainless-steel collection device was constructed to facilitate the collection of mites using carbon dioxide. Carbon dioxide cartridge with dust removing gun (dark arrow in left figure); carbon dioxide mite harvesting device (light elongated arrow in left figure); stainless steel canisters (light arrows in right figure); canister attachment device with wire mesh (dark arrow in right figure); entry port for carbon dioxide (dark elongated arrow in right figure).

Carbon Dioxide Concentration

A "HOBO MX" carbon dioxide Data Logger (MX1102; Onset Computer Corporation, Bourne, Massachusetts, USA) calibrated to the ambient conditions of the room was used to estimate the concentration of carbon dioxide introduced to the bees and mites before collection of *Varroa*. A 3.2 mm hole was placed in a 40-dram plastic vial and placed over the HOBO carbon dioxide sensor.

Mite Collection by the Sugar Shake Method

The sugar shake method was conducted in the manner outlined by Macedo et al. (2002) and Dietemann et al. (2013) with some modification described in Egekwu et al. (2018). Three hives were selected, just as was for the carbon dioxide collections. About 100 to 200 adult bees were gently brushed off frames into a 470 mL glass Mason jar (Ball® wide mouth pint, Walmart, Gainesville, Florida, USA). The jar lid was replaced with stainless steel mesh wire with 3 openings per cm (Gerald Daniel Worldwide, Houston, Texas, USA). The jar containing the bees was capped, and about 7 grams of powdered sugar (Publix, Lakeland, Florida, USA) was added through the mesh opening. The jar was gently agitated to cover the bees in sugar entirely and allowed to sit for 1 min. After the elapsed time, the jar was inverted over a container of water and shaken for about 1 min. The sugar sank or dissolved into the water while the mites floated on the water. Mites were retrieved using a fine paintbrush (the mites attached to the paintbrush when lightly touched), and placed on a moistened paper towel that was folded and transported to the lab for later use. After the mites were collected, the jar was opened, and the bees were introduced back into their hive.

HARVESTING BEE PUPAE AND *VARROA* SURVIVABILITY

Mite survivability was measured for mites collected using the carbon dioxide and sugar shake methods. The mites were maintained

on pupae in vitro with the *Varroa* maintenance system (Egekwu et al. 2018). Honey bee worker pupae (white-eyed) collected from apiaries maintained at the University of Florida Honey Bee Research and Extension Laboratory and the USDA-ARS Center for Medical and Veterinary Entomology, both located in Gainesville, Florida, USA, served as a food resource for the mites. The pupae were collected carefully to avoid damaging them. Each pupa was lifted with a pair of 0.13 cm, narrow-tip featherweight forceps (Bioquip, Rancho Dominguez, California, USA) from its open brood cell and placed in its own clear "00" gelatin capsule (Healthy Life Supply, Santa Ana, California, USA). Pupae were checked for traces of cuticular melanization, which is suggestive of injury in these insects (Lambrechts et al. 2004; Lee et al. 2008; Prokkola et al. 2013). Mites considered active were collected from the moist paper towel and introduced into the gelatin capsules containing bee pupae, 1 mite per pupa, using a Taklon size 0 paintbrush (Toray Chemical Co., Osaka, Japan). The mites freely crawled onto the pupae and the capsules were closed (Fig. 2). The replicate schedule for the study was: 2 collection methods (carbon dioxide and sugar shake) × 40 single bee and single mite combinations (each in a gel capsule) per collection method × 3 trials = 240 gel capsules total. The assay was run for a period of 6 d or until 100% mortality, as determined by visual inspection of the gel capsule. Unresponsive or moribund mites, determined by gentle prodding with a brush, were documented as dead.

STATISTICAL ANALYSIS

The impact of the collection method (carbon dioxide and sugar shake) on 6 d mite survival was determined for each trial individually, and then the 3 trials were averaged together using a Student's *t*-test (SAS 2013).

Results

CARBON DIOXIDE CONCENTRATION

The measurement range of the HOBO MX carbon dioxide logger was 0 to 5,000 ppm. The accuracy ± 50 ppm ± 5% of reading at 25 °C, < 70% RH, and 1,013 mbar. Our data show that honey bees and mites were being exposed to about 3,400 ppm (± 45.33) carbon dioxide using the device we developed (elevation of about 54 m).

MITE COLLECTIONS BY CARBON DIOXIDE AND SUGAR SHAKE METHOD

On average (across the 3 trials), mites collected using the carbon dioxide method had significantly lower survival (3.9 ± 0.3 d with 66% mortality at 6 d) than did mites collected using the sugar shake method

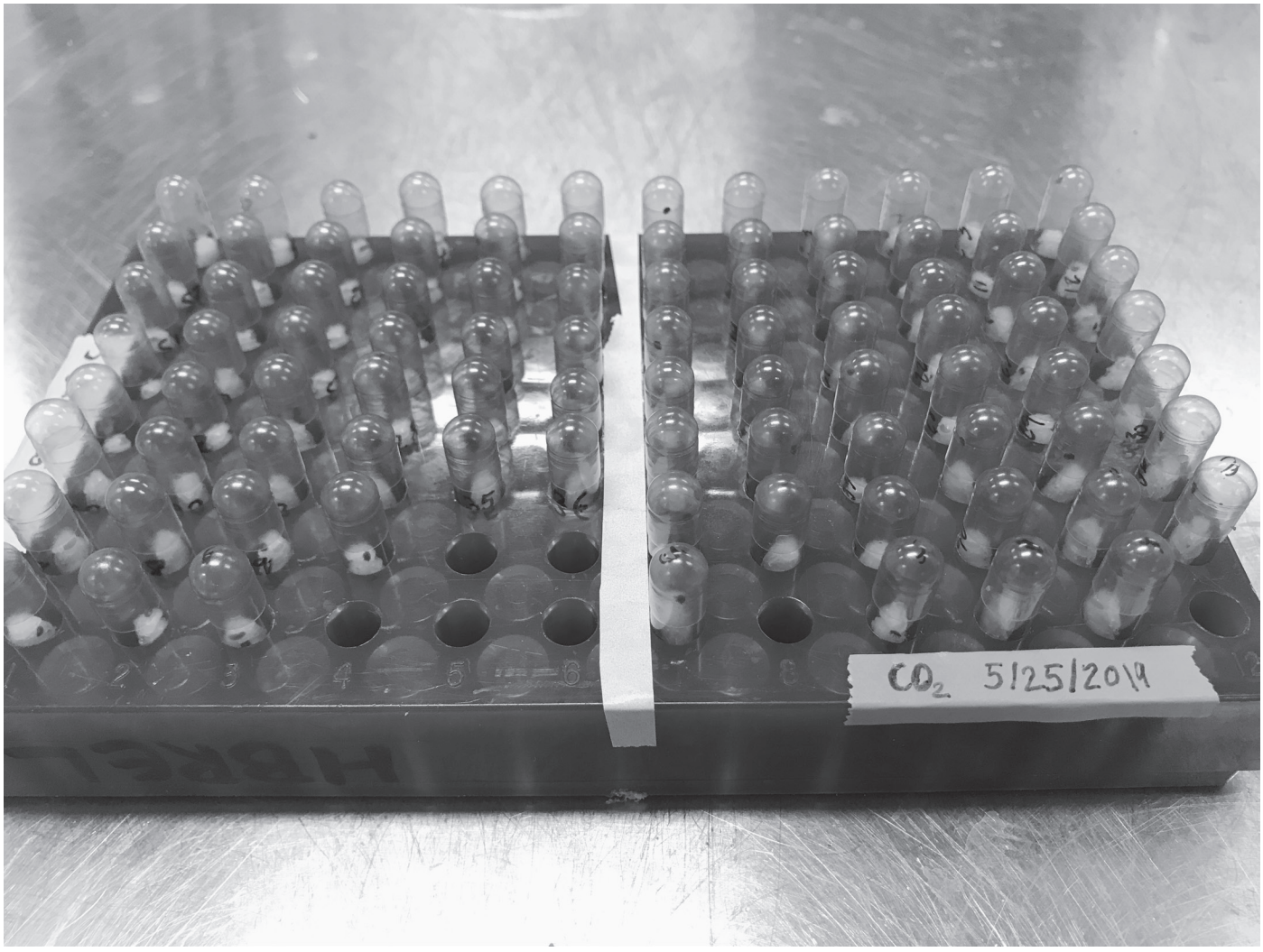

Fig. 2. *Varroa* maintenance system – single pupal honey bees are placed into gelatin capsules with single adult female *Varroa destructor*.

(5.3 ± 0.1 d with 20% mortality at 6 d) (T = 34.6; df = 1; $P < 0.0001$, n = 240). This trend was true across all 3 trials when analyzed separately (Fig. 3).

Discussion

Several in vitro studies have been conducted using *Varroa* harvested via different methods depending on the experimental goals. Among the methods used, mites collected manually for use in in vitro systems had reasonable reproductive rates (Nazzi & Milani 1994; Donze et al. 1996; Jack et al. 2020). The powdered sugar collection method also has been useful for collecting *Varroa* for in vitro studies focusing on the survival of non-reproductive mites (Egekwu et al. 2018; Posada-Florez et al. 2018). Despite these studies, nobody to our knowledge has explored the effect of carbon dioxide-collection on *Varroa* survival in vitro.

Selecting the best method to collect *Varroa* for use in in vitro research is difficult. *Varroa* are known to live and reproduce within honey bee colonies (Anderson & Trueman 2000); therefore, collection from hives and subsequent maintenance of the mites in the laboratory has been challenging (Egekwu et al. 2018). In this study, we tested the impacts of the collection methods (carbon dioxide and sugar shake) on *Varroa* mortality in a 6 d test. Because we focused on harvesting non-reproductive mites, manual collection of reproductive mites from capped brood cells was not considered. Manual collection is described already in the literature (Donzé & Guerin 1994; Donzé et al. 1996; Dietemman et al. 2013). Furthermore, non-reproductive mites may be manipulated into reproducing in vitro (Egekwu et al. 2018), making collecting them rather than reproductive mites already in capped brood cells appealing.

Our data provide some insight for future research into the collection of *Varroa* for in vitro studies. Our data suggest that mites collected using the sugar shake method will survive longer in in vitro studies than will mites collected using carbon dioxide. Macedo et al. (2002) documented higher survival of mites harvested using the powdered sugar method than when using other inert dusts, but not compared to mites harvested manually from capped brood cells. Nevertheless, we tested only a small range of carbon dioxide concentrations, so we cannot suggest that all carbon dioxide collections are equally harmful to mites.

There was a natural range of carbon dioxide concentrations produced using the device we developed. This occurred because it is hard to standardize the release of carbon dioxide from the applicator we used. It is possible that carbon dioxide may still be used to collect *Varroa*, but the useful concentration would be below that which we tested.

In conclusion, *Varroa* collected via the sugar shake method survived longer than those collected using carbon dioxide. Strategies that enhance *Varroa* survival the first few critical d of in vitro tests are sorely needed as the scientific community moves toward in vitro rearing programs for *Varroa*. We speculate that the use of powdered sugar does not interfere negatively with *Varroa* physiology. *Varroa* collected this way have been observed to survive > 4 d (Macedo et al. 2002) and 5

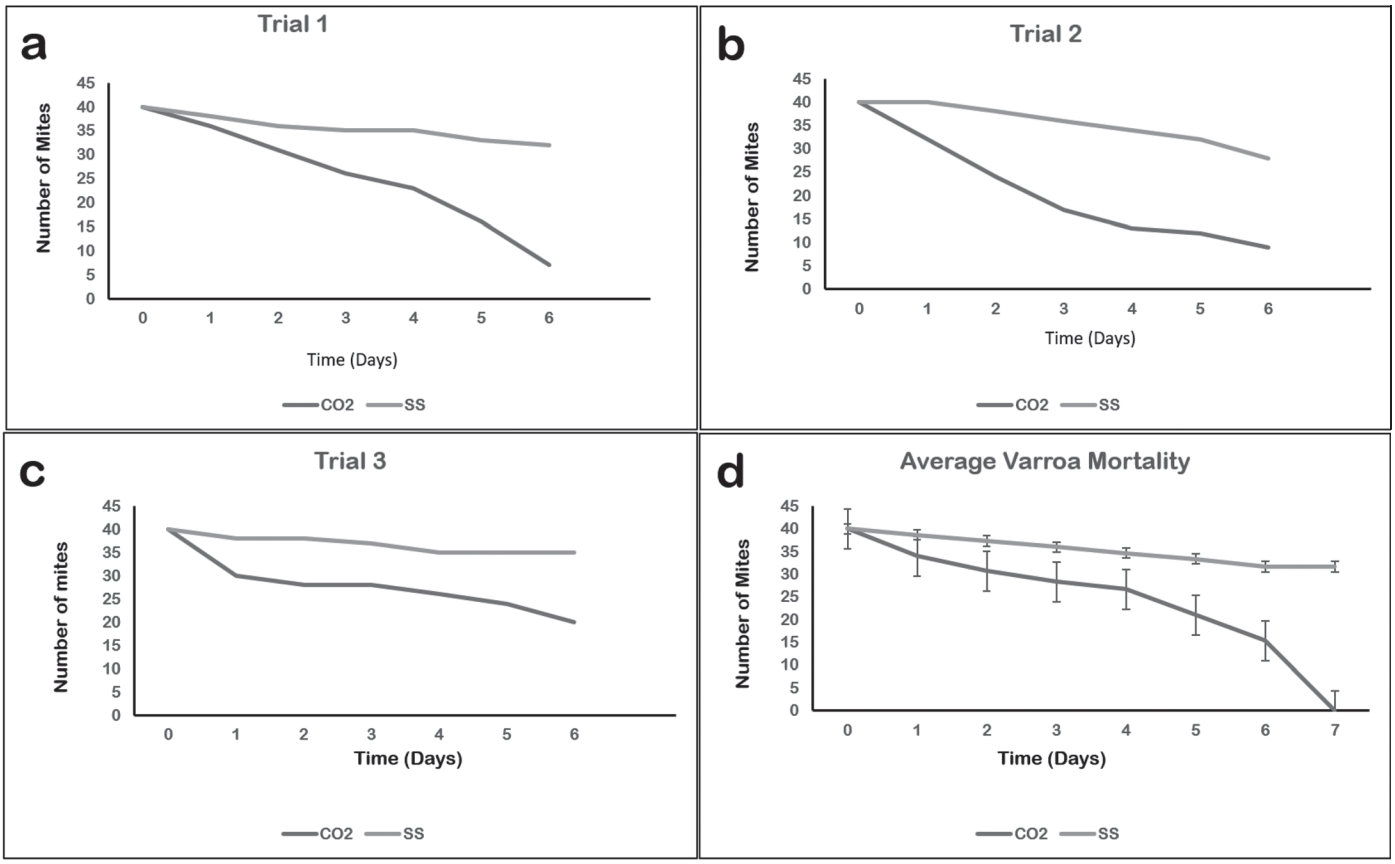

Fig. 3. Trial 1 (a), 2 (b), and 3 (c) survival (± SE) over time (d) of adult *Varroa destructor* after placement upon pupal hosts. N = 40 pupa per *Varroa* combinations per collection method (sugar shake – ss; carbon dioxide – CO2) per trial. Box (d) represents mean (± SE) survival over time (d) of adult *Varroa destructor* after placement on pupal hosts. N = 3 trials per data point. There was a significant difference in mite survival between mites collected both ways for Trials 1 (T = 19.21; df = 1; $P < 0.0001$), 2 (T = 22.61; df = 1; $P < 0.0001$), and 3 (T = 15.59; df = 1; $P < 0.0001$), and for the average across all 3 trials (T = 34.58; df =1; $P < 0.0001$), with mite survival always highest for mites collected via the sugar shake (ss) method.

to 7 d (personal observation) in a paper towel lightly wetted with powdered sugar/water solution left at room temperature and maintained without food. Carbon dioxide, on the other hand, clearly impacts mite survival, as has been shown for other organisms (Czekonska 2009). Our results demonstrate that individuals studying *Varroa* should consider how the collection method impacts *Varroa* behavior and performance in a study.

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