The defensive behavior of Indiana mite biting honeybees against *Varroa destructor* and the structure of mandibles

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Abstract:

The honeybees (*Apis mellifera*) are the most important managed pollinator for sustainable agriculture and our ecosystem. However, the managed honeybee colonies in the United States experience 30-40% of losses annually. Among all the biotic stressors, the parasitic mite *Varroa destructor* is considered as one of the main pests for colony losses. The mite biting behavior as a *Varroa* tolerant or resistant trait has been selected in the State of Indiana for over a decade (13 years). A survey of damaged mites from the bottom of a colony can be used as an extended phenotype of the mite biting behavior to evaluate a colony. On average 37% of mites sampled were damaged or mutilated from the breeding stocks of 59 colonies of mite biters of Indiana, which is significantly higher than 19% of damaged mites in commercial colonies from the Southern United States. No significant difference was detected between the damage of missing legs between breeding stocks and commercial colonies. In addition, the morphology of pollen forager worker mandibles was compared between two populations via X-ray micro-computed tomography using six parameters, and a difference was detected in one parameter. Our results showed the novel scientific evidence to explain the potential defensive mechanism against *Varroa* mites via potential structural changes in worker mandibles.

Introduction

The infestation of ectoparasitic mites *Varroa destructor* is the primary concern for honeybee (*Apis mellifera*) colony losses since these mites were introduced to the United States since 1987 (Guzmán-Novoa et al., 2010; Le Conte et al., 2010; Nazzi and Le Conte, 2016). Mature *Varroa* females are 1.1x1.5 mm, and the males are 0.8 x 0.7 mm in sizes. The majority of a mite's life cycle happens in the brood cells including developing stages of egg, six-legged larva, protonymph, deutonymph, and adult (Bailey, 1968; Genersch, 2010). *Varroa* mites can weaken, deform, and decrease the lifespan of the infested honeybee colony because mites feed on the honey bee pupae and adults, transmit viral pathogens, cause morphological and behavioral defects on bee development and behavior (Le Conte et al., 2010). Recent scientific evidence showed that *Varroa* mite feed on the fat body of adult bees (Ramsey et al., 2019). Furthermore, *Varroa* mite is an effiective vector to transmit honey bee viruses within the honeybee colony (Di Prisco et al., 2011; Wilfert et al., 2016).

The European Honeybee (*A. mellifera*) has developed defensive behavior to keep the mite population low by grooming, biting, and hygienic behaviors against *Varroa* mites (Arechavaleta-Velasco and Guzmán-Novoa, 2001; Guzman-Novoa et al., 2012; Ruttner and Hanel, 1992; Spivak, 1996; Tsuruda et al., 2012; Villa et al., 2017). The biting behavior, also considered a type of grooming behavior, enables worker bees to bite and remove adult mites from their bodies (Peng et al., 1987; Pritchard, 2016; Ruttner and Hanel, 1992). Colonies selected by instrumental insemination or open mating with feral colonies on mite biting behavior potentially have greater fitness over subsequent generations. Field reports showed that Indiana mite biters from Purdue University are being selected in the past decade, and dramatic increases in the average proportion of damaged mites in their breeding population (Andino and Hunt, 2011; Hunt et al., 2016; Morfin et al., 2019). However, to date, there is no report to compare the Indiana mite biter honeybee stocks to the commercial population in the United States.

The breeding of mite resistant bees is critical to maintaining sustainable apiculture for local pollination, food, and crop productivity (Oddie et al., 2017). Modern beekeeping and breeding techniques of instrumental insemination enable artificial selection effects on honeybee

queens and colonies (Meixner et al., 2010). Breeding efforts have been made in different continents and countries to select mite tolerance or resistant traits (Büchler et al., 2010; Rinderer et al., 2010; Spivak, 1996; Villa et al., 2017). Mite resistant bees will assist beekeepers in managing the growing chemical miticide-resistance problems and play a critical role in promoting sustainable agriculture practices (Hamiduzzaman et al., 2017; Kanga et al., 2016). In the past, research efforts for breeding are focused on different bee breeding stocks to improve colony health, including Russian bees, *Varroa* Sensitive Hygienic bees, Minnesota Hygienic bees (Buechler et al., 2010; Danka et al., 2011; Guarna et al., 2017; Guarna et al., 2015; Rinderer et al., 2010; Spivak, 1996; Villa et al., 2017).

Honeybee mandibles are considered to be the main mouthpart that worker bees use to bite or chew the parasites, including mites and wax moths in the colony (Ruttner and Hanel, 1992). Chemical analysis revealed 2-Heptanone was secreted from the mandibles, acting as an anaesthetic on wax moth larva and *Varroa* mites (Papachristoforou et al., 2012). Micro-X-ray-computed tomography (MicroCT) is a technology with a fast 3-D scanning in satisfactory spatial resolution without complicated and lengthy sample preparation procedures. In the past, this technique has been used to study the brain anatomy and evolution of bees, ants, and other insects (Coty et al., 2014; Larabee et al., 2017; Li et al., 2011; Ribi et al., 2008). However, there is no report to use MicroCT to demonstrate the shape of honeybee mandible in fine details.

In this study, we hypothesized that the Indiana mite biter breeding stocks have a higher level of mite biting behavior than commercial bee colonies. Our goal is to characterize the behavioral and morphological capacity for the mite biting behavior. Total number of mites, the percentage of damaged mites as the parameter for mite biting behavior, and the number of mite legs missing per colony were reported. In addition, we investigate the mechanism of mite biting behavior by examining the 3-dimensional shapes of mandibles between Indiana mite biter colonies and commercial colonies from the Southern United States (mainly the state of Georgia).

Material and Methods

Honeybee colonies

Fifteen colonies from five commercial source were sampled from beekeepers who bought their package colonies in 2018 originally from the State of Georgia (from five different commercial providers). Mites were collected in September of 2018 from different areas in the State of Ohio (Figure 1a – 1 colony from Defiance (Defianace1), OH; and b – 14 colonies from Bellbrook (NA1, NA2, NA3, NA5, NA6), Beavercreek (PBJohn1, PBJohn2), Cedarville (Dan4), and Wilberforce (AB1, CSU23, CSU24, CSU32, CSU51, CSU52) OH). Total of 59 colonies from Indiana mite biter honeybees were sampled at Lafayette, Indiana (Figure 1c, colony numbers are in the supplemental table 1). Mite samples were collected over a 5-day period as fresh mites from each colony at Purdue University's main apiary in the fall of 2018. Some colonies sampled twice during the fall season. Seven colonies of Wright Patterson Air Force Base (WPAFB) Huffman prairie site housed seven virgin queens from Purdue stock colonies and then open-mated with drones from feral colonies near WPAFB (within two miles).

For all of the worker bee samples of the mandible scanning, pollen foragers returning to the hive (either commercial or Indiana mite biter colony) entrance were collected using an insect vacuum (Cat: 2820GA, BioQuip). Then all bees were frozen on dry ice, transported back to the lab and kept in -80 freezer. At least 10 foragers were collected per colony per site. Indiana mite biter bee samples were collected in June 2018. Commercial bee samples were collected in July 2018.

Varroa Mites

Three groups of mites were compared: 1) commercial (comm), 2) mite biters (from Indiana), and 3) mite biter open mated (at WPAFB). Figure 2 showed one example of a worker bee biting a *Varroa* mite on the top of a hive. Mite samples of Indiana mite biters were collected following the previously described method (Andino and Hunt, 2011). For commercial bees and mite biter open mated in Ohio, mite samples were collected as previously reported (Andino and Hunt, 2011). A small paint brush was used to remove mites from the bottom boards to a plastic disposable cup with a lid (volume 1oz). Mite samples stayed in a -20 freezer overnight. Each mite was carefully glued onto a glass microscope slide (25 x 75 x 1mm, Globe Scientific Inc.) by a small paint brush. Slides were examined under a light microscope (Zeiss STEMI 580) with a magnify degree of 50x. Colonies with 15 or more mites sampled within a 5-day period were included in the data analysis. Mites collected in Ohio were examined for any missing legs (range

from 1 to 8) as visible damage in the view (Figure 3). The total number of mites sampled per colony and the number of damaged mites were compared among all three groups (comm, IN mite biters, and mite biter open mated). The number of missing legs were compared between commcial colonies and mite biter open mated colonies. Immature mites and empty shells of mite body were excluded.

MicroCT Scanning

From commercial colonies, five bees (five pairs of mandibles) were scanned from two colonies (three bees from colony PBJohn2 and two bees from colony Dan4). For the Indiana mite biters, nine bees from three colonies (three bees from colony 5, four bees from colony 15, and two bees from colony 41) were scanned. The scanning process was performed at the Center for Electron Microscopy and Analyses (CEMAS) of Ohio State University (Columbus, OH) using a HeliScan (FEI, Thermal Fisher, USA) for 3-dimensional imaging. A pair of mandibles was fixed to thin wooden posts (r = 1 mm and h = 148mm) using super glue to fit in the HeliScan. The scanning parameters were as follows: isotropic voxel size = 2.564 m per pixel; voltage =60 or 80 KV, current = 80 or 46 micro A; helical scan using space filling trajectory reconstruction (Kingston et al., 2018) without any filter, and 1440 X-ray raw images. The software AVIZO for FEI systems (version 9.4) was used to quantify the measurements (Height, Length, Width, Small Edge, Long Edge, and the Span of spike area) for each scanned sample.

Data Analysis

The average number of mite samples per colony was compared among three groups, commercial (comm) bees, Indiana mite biters, and the mite biters open mated at WPAFB. If a colony was sampled twice in the fall season, the average number of mites collected was used for statistical analysis. The percentage of damaged mites over total sampled mites was transformed using positive square root for normal distribution. One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference) Test calculated for comparing multiple treatments test was used for the difference among means of different populations as to the total mite numbers, mite biting rate, damage of legs, and parameters of mandibles. The online tool Interactive Dotplot (Weissgerber et al., 2017) was used to generate all boxplots.

Results

The behavior of mite biting or grooming refered to here describes how a worker bee uses its two forlegs and two mandibles of its mouthpart to attack a *Varroa* mite in a honeybee colony. To characterize the mite damage, we categorized the mites observed as four different types: (A) mature adult female mites in dark brown colors with no damage and all eight legs (Left 1, 2, 3, 4, Right 1, 2, 3, 4) are present, (B) damaged adult female mites with legs (in this case, four legs Left 1, 2, 3, 4) missing, (C) young adult female mites which are pale in color and are not counted as a damaged adult mite, (D) mites with partial body missing (Figure 2). Mite samples similar to C type are excluded because no damage was detected by worker bees' grooming or biting behavior.

To evaluate the total mite population, we collected all the *Varroa* mites that appeared on the bottom board of each colony from each commercial colony (15 colonies, $N_{mite} = 886$), mite biter colony (59 colonies, $N_{mite} = 3{,}390$), and mite biting open mated colony (7 colonies, $N_{mite} = 569$). There was no significant difference or significant interaction detected among the commercial colonies, mite biter colonies, and mite biter open mated colonies (one-way ANOVA of three independent treatments, $F(2, 80) = 1.1992$, $p = 0.3069$, Figure 3).

To assess the mite biting behavior or grooming behavior, we surveyed all the damaged mites collected from the bottom board of each colony from all three groups (commercial colonies, $N_{mite} = 172$, mite biter colonies, $N_{mite} = 1{,}201$, mite biter open mated colonies $N_{mite} = 199$). The mean of the percentage of damaged mites per colony among three treatments (commercial, mite biter, and mite biter open mated) after transformation are 39.80%, 60.22%, and 60.14%. One-way ANOVA analysis suggests that one or more groups are significantly different ($F(2, 80) = 31.3785$, $p = 1.0031e\text{-}10$). The Tukey HSD test identified that the levels of mite biting behavior or grooming behavior in the mite biter are significantly higher than the commercial colonies ($Q = 11.0866$, $p < 0.01$, Figure 4). Levels of mite biting behavior or grooming behavior in the mite biter open mated colonies are significantly higher than the commercial colonies ($Q = 6.9771$, $p < 0.01$, Figure 4). Levels of mite biting behavior or grooming behavior between mite biter and mite biter open mated colonies are not significantly different ($Q = 0.0304$, $p > 0.05$, Figure 4). Commercial bees have the lowest mite biting behavior among all three groups.

To evaluate the damage of mites and potential difference between commercial colonies and mite biter open mated colonies, we counted the number of legs missing from each damaged mite. Our result showed no difference regarding the average number of legs missing per mite between these two groups ($Q = 2.4278$, $p > 0.05$, Figure 5).

To compare the morphology of mandibles, six different parameters (Figure 6) including the length (L), the width (W), the height (H), long edge (LE), short edge (SE), and span of the spine area (S) are measured from the microCT data between commercial colonies and mite biter colonies. Statistical analysis (ANOVA) between these two groups showed that the long ege (LE) of mite biter colonies are significantly short than the commercial colonies ($F = 5.7762$, $p = 0.0256$, Figure 7). There is no significant difference of the other five parameters between two groups (Figure 7), but in the length, height, short edge and span of the spine area, we noticed a consistent trend of a smaller value in mite biter colonies.

Discussion

We have investigated the *Varroa* mite population and the mite biting behavior among mite biter colonies, mite biter open mated colonies, and commercial colonies in the U.S. Also, we evaluated the difference in the shape of bee mandibles between mite biter honeybee stock and commercial colonies. The mite biter colonies displayed a higher level of mite biting behavior than the commercial colonies and the difference in the long edge of their mandibles may provide a potential explaination of a physical mechanism to mutilated mites using mandibles.

Previous research on grooming behavior and damaged mites in *Apis* showed that grooming behavior is a selected trait in naturally mite-resistant colonies (Arechavaleta-Velasco and Guzmán-Novoa, 2001; Boecking and Ritter, 1993; Fries et al., 1996; Peng et al., 1987; Russo et al., 2020). As one of the defensive behaviors of honey bees for parasitic *Vaoora* mites, the mite biting or grooming behavior can be used as a parameter to selection honey bee stocks for *Varroa* mite resistance (Hunt et al., 2016; Morfin et al., 2019; Pritchard, 2016; Rinderer et al., 2010; Spivak, 1996).

Our present comparisons between commercial and mite biter bees support the value of selection of mite biting behavior in *A. mellifera*. With our evidence of freshly damaged mites, we

provided a strong argument that workers of *A. mellifera* are able to amputate the legs of *Varroa* mites as described before (Ruttner and Hanel, 1992). Damaged mites collected from the bottom board of each colony within a five-day time ensured the observed damage on mites was fresh. It is possible for beekeepers to record the proportion of damaged mites from the bottom board of colonies and create a collaborative regional effort for a selection in a region (Bienefeld et al., 1999; Hunt et al., 2016).

As a defensive response, honeybees use biting or grooming behavior to decrease the infestation of a mite population in the colony. It is known that worker bees can use their mandibles to multilate or damage *Varroa* mites from reports published before (Ruttner and Hanel, 1992). Further studies showed that certain chemical such as 2-Heptanone can be released from worker's mandibles during a bite to anaestheze parasites including *Varroa* mites and wax moths in honeybee colonies (Papachristoforou et al., 2012). Although there are reports on the mite biting behavior, there is no report on the underlying mechanism for this behavior. Our results provided empirial evidence on the changes of the structure of mandibles such as long edge which can be an explanation for the mechanism of biting behavior. In addition, our data showed that mite biters are under selelction which may lead to the structure changes for mite resistance. Eastern honeybees (*A. cerana*) are original hosts of *Varroa* mites, and have evolved to be *Varroa* mite resistant now in Asia. Their body size are slightly smaller than *A. mellifera* (Peng et al., 1987; Yue et al., 2018).

Our data indicate the clear trend that mite biters are evolving a behavioral adaptation to the parasitic mite *V. desctructor*. The sensitivity of workers to the mites in colonies selected for mite-biting behavior is higher, and they perform higher level of mite biting behavior compared to commercial colonies given similar numbers of mite population in all colonies. However, this study cannot compare these stocks ability to reduce mite populations because they were in different geographic locations and had different management histories.

The worker bee's ability to detect mites may be based on the olfactory mechanisms considering the mite biting behavior happens in the dark hive most time. The stocks of mite biters may show a better capability to detect and recognize mites as pest than non-selected commercial colonies. There may be gradual changes in the relationship between *A. mellifera* and *Varroa* mites. There is a potential change in the biting mechanism of mite biters, which Asian

honeybees displayed in previous research(Peng et al., 1987). Compared to the low frequency of removing mites or low success in clearning the mites from commercial-source colonies, we observed an improvement of these abilities in mite biter colonies.

Even though there is evidence for variation in the mite biting or grooming behavior in different genotypes (Guzman-Novoa et al., 2012), more knowledge is needed on the genetic architecture and the pattern of inheritance of this behavior for honeybee breeding and selections. With rapid development of new sequencing technologies and genome editing tools, genome-wide marker-assistant selelction may be applied in the future for honeybee breeding.

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Author contributions

H.L.-B. conceived and designed experiments; J.S., X.L.C., K.G. & H.L.-B. collected samples & data. J.S. and H.L.-B. performed data analysis and wrote the paper. All authors approved the final manuscript.

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Figure and Table legend

Figure 1. The map of locations for sample collection from the State of Ohio and Indiana. (a site) Defiance, OH; (b site) Green county including cities of Bellbrook, Beavercreek, Cedarville, and Wilberforce, OH. (c site) West Lafayette, IN for Indiana mite biter colonies.

Figure 2. Images of damaged *Varroa* mites from our sampled colonies. A: matured mite with no damage, Labeled numbers of 1-8 indicate eight legs of the *Varroa* mite; B: a damaged mite with legs missing; C: young mature mites; D: a matured mite with a missing body part. Arrows indicated the damaged legs or the body part.

Figure 3. The box plot of the average number of mites collected per colony among three groups, commercial colonies (Comm, $N_{colony} = 15$), Indiana mite biters (mite biter, $N_{colony} = 59$), and mite biter open-mated colonies (mite biter open mated, N = 7). The open circle is the outlier of colonie. $F(2, 80) = 1.1992$, $p = 0.3069$. The p-value corresponding to the F-statistic of one-way ANOVA suggests that all three treatments are no significantly different.

Figure 4. The violin plot of the percentage of damaged mites identified versus total mites collected per colony among three groups, commercial colonies (Comm, $N_{colony} = 15$, $N_{mite} = 172$), Indiana mite biters (mite biter, $N_{colony} = 59$, $N_{mite} = 1{,}201$), and mite biter open mated colonies (mite biter open mated, $N_{colony} = 7$, $N_{mite} = 199$). The mean of the percentage of damaged mites per colony among three treatments (commercial, mite biter, and mite biter open mated) after transformation are 39.80%, 60.22%, and 60.14%. One-way ANOVA analysis suggests that one or more groups are significantly different ($F(2, 80) = 31.3785$, $p = 1.0031e\text{-}10$). The Tukey HSD test identified that the levels of mite biting behavior or grooming behavior in the mite biter are significantly higher than the commerican colonies ($Q = 11.0866$, $p < 0.01$). Levels of mite biting behavior or grooming behavior in the mite biter open mated colonies are significantly higher than the commercial colonies ($Q = 6.9771$, $p < 0.01$). Levels of mite biting behavior or grooming behavior between mite biter and mite biter open mated colonies are not significantly different ($Q = 0.0304$, $p > 0.05$).

Figure 5. The box plot of the average number of legs missing per mite per colony among two groups, commercial colonies (Comm, $N_{colony} = 15$) and mite biter open-mated colonies (mite biter open mated, $N_{colony} = 7$). The open circle was outliers. Our result showed no difference regarding to the average number of legs missing per mite between two groups ($Q = 2.4278$, $p > 0.05$).

Figure 6. Six parameters of the scanned mandible are listed from the microCT image. L- length, W –width, H-height, SE-short edge, LE – long edge, S-span of spine area.

Figure 7. The box plot of the six parameters of mandible morphology between two groups, commercial colonies (Comm, $N_{mandible} = 10$) and mite biters (mite biter, $N_{mandible} = 15$). Each filled circle is each data point. The p-value corresponding to the F-statistic of one-way ANOVA suggests that two treatments are not significantly different in length, width, height, short edge, the span of spine area, but significantly different in long edge ($F(1, 21) = 5.7762$, $p < 0.05$). There is no significant difference of other five parameters between two groups, but in the length,

height, short edge and span of the spine area, we noticed a consistant trend of smaller value in mite biter colonies.

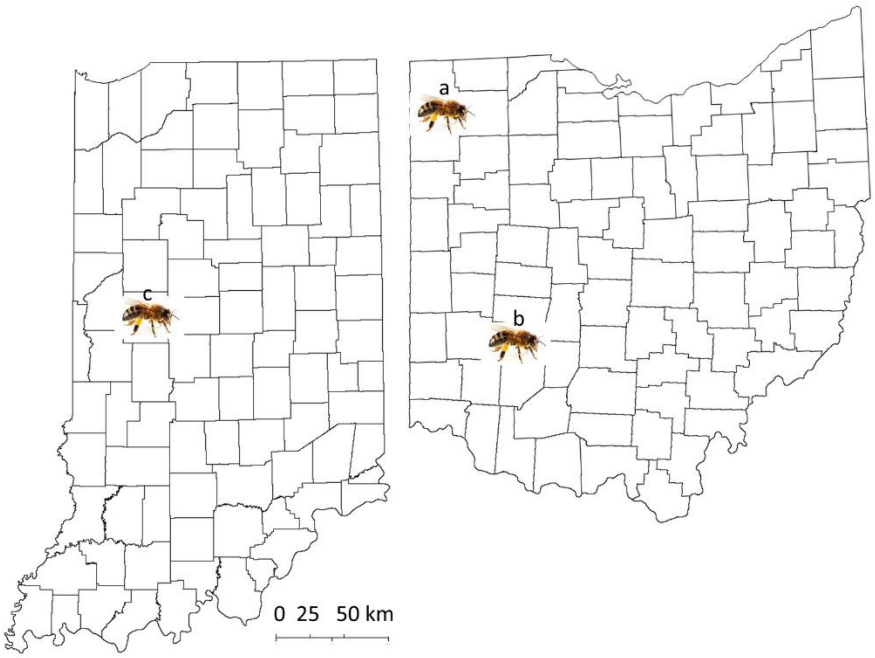

Figure 1

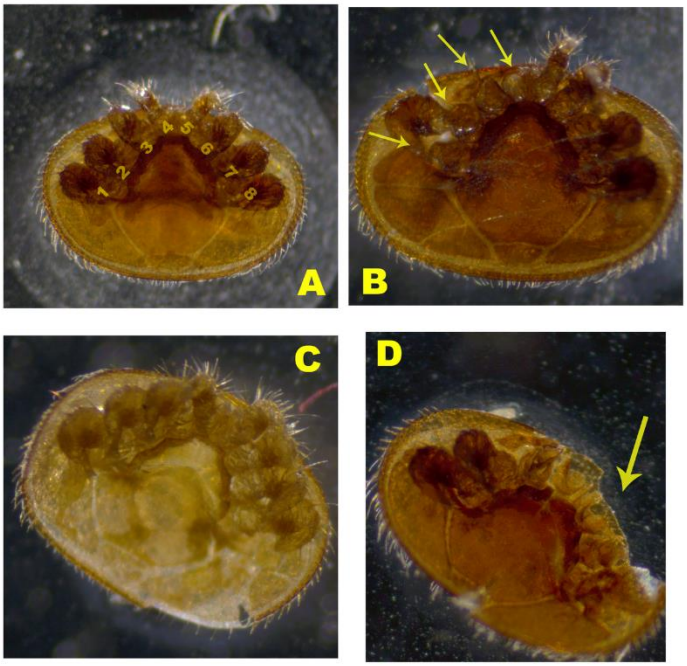

Figure 2

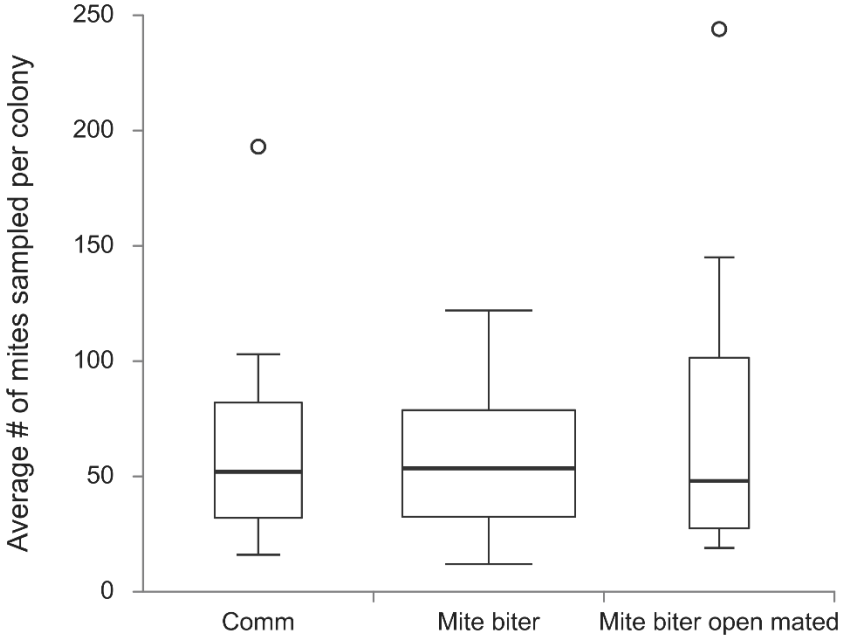
Average # of mites sampled per colony
250
200
150
100
50
0
Comm
Mite biter
Mite biter open mated

Figure 3

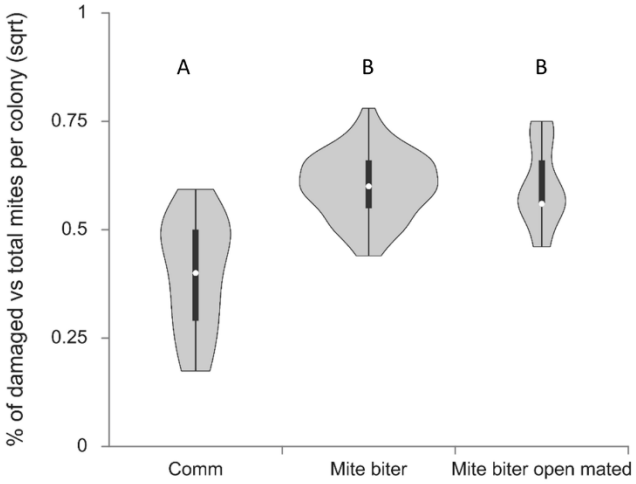
% of damaged vs total mites per colony (sqrt)
1
0.75
0.5
0.25
0
A
B
B
Comm
Mite biter
Mite biter open mated

Figure 4

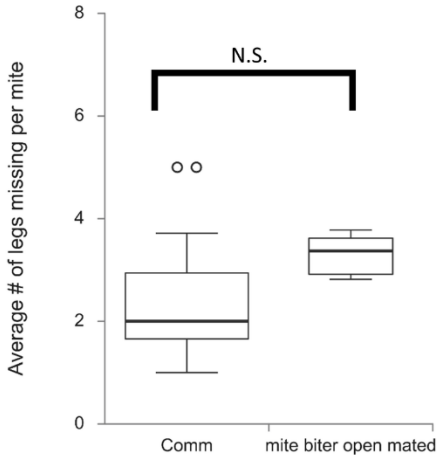
N.S.
Average # of legs missing per mite
8
6
4
2
0
Comm
mite biter open mated

Figure 5

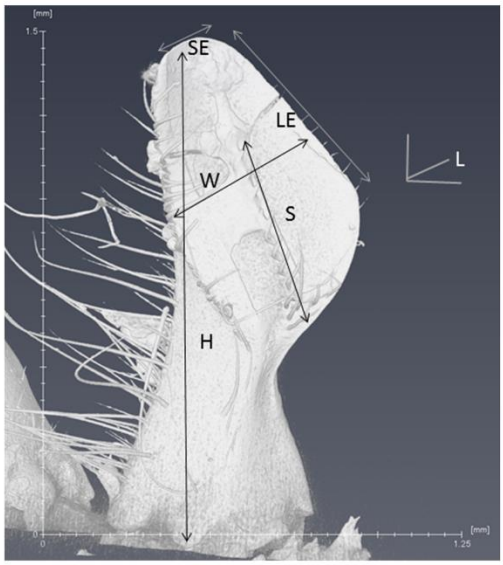

Figure 6

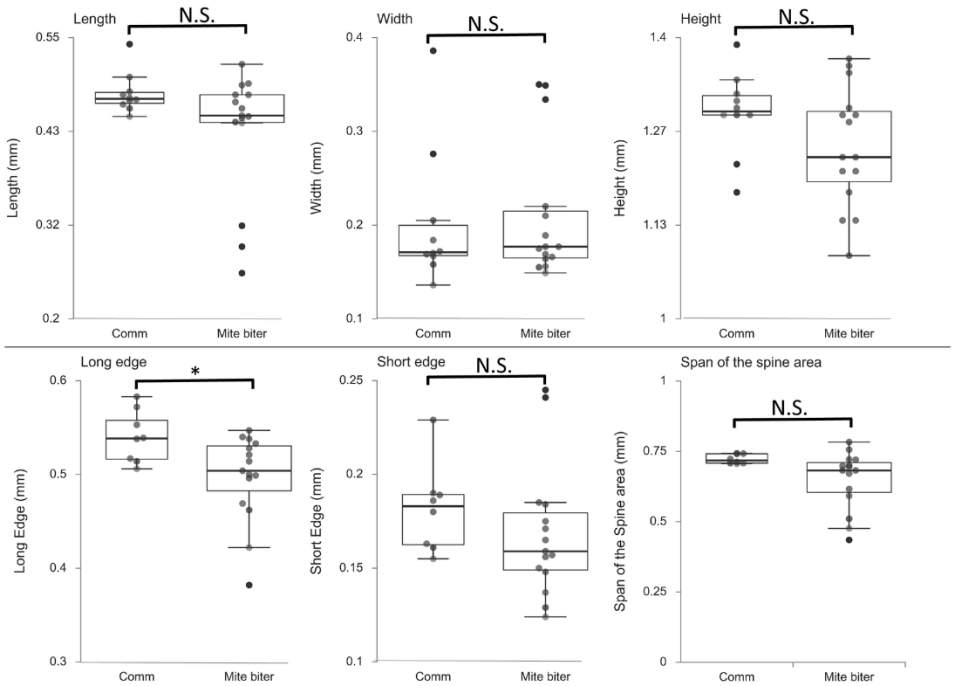
Length
N.S.
Length (mm)
0.55
0.43
0.32
0.2
Comm
Mite biter
Width
N.S.
Width (mm)
0.4
0.3
0.2
0.1
Comm
Mite biter
Height
N.S.
Height (mm)
1.4
1.27
1.13
1
Comm
Mite biter
Long edge
*
Long Edge (mm)
0.6
0.5
0.4
0.3
Comm
Mite biter
Short edge
N.S.
Short Edge (mm)
0.25
0.2
0.15
0.1
Comm
Mite biter
Span of the spine area
N.S.
Span of the Spine area (mm)
1
0.75
0.5
0.25
0
Comm
Mite biter

Figure 7

Reference

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