ABSTRACT

Title of Thesis:	PATHOPHYSIOLOGICAL EFFECTS OF THE MICROSPORIDIUM NOSEMA CERANAE INFECTION ON WORKER HONEY BEES (APIS MELLIFERA).
	Serhat Solmaz, Master of Science, 2021
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Nosema ceranae is an important stressor in honey bee colonies all over the world. *N. ceranae* infection can potentially cause serious economic damage in the industry if left untreated. We designed two experiments to investigate the associations between *N. ceranae* infection and pathophysiological traits occurring in the honey bee body. First, we conducted a retrospective cohort study. We dissected and scored specific tissues of the bees and compared scores of bees coming and not coming from apiaries with high *Nosema* loads Next, we conducted a cage study. We inoculated a group of bees with *N. ceranae* spores and collected samples from cages at different points in time. By this, we found the exposure to *N. ceranae* increases the relative risk of certain pathophysiological traits. Pathophysiologies occurring in the bodies of infected bees over time help elucidate the progression of this disease in the bee body from the standpoint of pathophysiology. We found that *N. ceranae* exposure advanced the onset of some age-related pathologies.

PATHOPHYSIOLOGICAL EFFECTS OF THE MICROSPORIDIUM NOSEMA CERANAE INFECTION ON WORKER HONEY BEES (APIS MELLIFERA).

by

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Dedication

I dedicate this work to my family and friends. I must thank my mother, father, brother, and my sisters for always being available and supportive towards me. I must also thank all my friends both from United States and from Turkey for always putting a smile on my face and making me a happier person.

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Introduction

Honey bees (*Apis mellifera*) serve an important role in our agriculture and economy (Calderone, 2012). They pollinate a wide range of our economically important crops (Calderone, 2012). Following the mass colony die-offs during the winter of 2006-2007 (van Engelsdorp, Hayes, Underwood, & Pettis, 2008), however, colony health has become a growing concern among beekeepers. Reports show the annual losses have remained high (~30%) for the past decade (Kulhanek et al., 2017; Lee et al., 2015; Seitz et al., 2015; Spleen et al., 2013; Steinhauer et al., 2014). These losses are attributed to numerous factors such as pesticides, nutritional stress, parasite infections, and pathogens (including viral, bacterial and fungal infections), or different combinations of these and other drivers (Steinhauer et al., 2018).

The microsporidian *Nosema ceranae* is a fungal parasite (Adl et al., 2005; I Fries, Martín, Meana, García-Palencia, & Higes, 2006) that was first identified in the Asian honey bee (*Apis ceranae*) (Ingemar Fries, Feng, Da Silva, Slemenda, & Pieniazek, 1996). This parasite is widespread in European honey bees (*Apis mellifera*) around the world (Higes, Martín, & Meana, 2006). Similar to other Microsporidia, N.

ceranae can alter certain aspects of the host's physiology and behavior. Such changes include decreased ventriculus (midgut) tissue integrity (Dussaubat et al., 2012), immune response suppression (Antúnez et al., 2009; Aufauvre et al., 2014), altered regulation of pheromones(Goblirsch, Huang, & Spivak, 2013; Holt, Aronstein, & Grozinger, 2013; C. Mayack, Natsopoulou, & McMahon, 2015), and changes in energy metabolism (Christopher Mayack & Naug, 2009) of infected bees. Microsporidia lack the cellular mitochondria crucial for ATP production. They are, therefore, highly dependent on the host for ATP supply needed for their growth and reproduction (Cornman et al., 2009; Hacker, Howell, Bhella, & Lucocq, 2014; Keeling & Fast, 2002). *N. ceranae* is not an exception. They also exert an energetic stress on their host(Christopher Mayack & Naug, 2009), which might cause an altered energy metabolism in infected individuals. Previous studies showed that bees with *N. ceranae* infection have a higher sugar demand and consumption (Alaux et al., 2010; Martín-Hernández et al., 2011; Christopher Mayack & Naug, 2009; Naug & Gibbs, 2009).

While *N. ceranae* infection can harm individual bees, the cumulative effect of high rates of infection in the same colony has negative consequences for the colony overall. As social insects, honey bees have a well-developed in-hive age dependent division of labor (temporal polyethism)(Huang & Robinson, 1996). Vitellogenin (Vg) is a glucolipoprotein that plays an important role in temporal polyethism, and is produced and stored within the fat body of honey bees (Paris, El Alaoui, Delbac, & Diogon, 2018). Multiple studies demonstrate that the genes responsible for Vg

production are expressed at lower levels in honey bees infected with *N. ceranae* (Antúnez et al., 2009; Garrido et al., 2016; Goblirsch et al., 2013; Paris et al., 2018). Low levels of Vg are thought to be responsible for artificially aging bees and causing precocious foraging (Marco Antonio, Guidugli-Lazzarini, Do Nascimento, Simões, & Hartfelder, 2008). This suggests *N. ceranae* can also have adverse effects on the honey bee health at a colony level by altering the labor division (advancing the onset of foraging, shortening the lifespan of foragers) (Woyciechowski & Moroń, 2009). *N. ceranae* infection is also linked to reduced honey production and depopulation in infected colonies (Botías, Martín-Hernández, Barrios, Meana, & Higes, 2013; Martín-Hernández et al., 2007).

Worker bees usually perform different and predictable colony tasks as they age, concentrating on in-colony tasks when they are younger and outside tasks when they are older. Through this task differentiation, worker bees are able to respond to deficits and excesses depending on their colony's needs. This plasticity is expressed through the delay or reversion of their labor-specific behavioral developments and physiological traits (Huang & Robinson, 1992; Münch & Amdam, 2010). Although this ability to show resilience is beneficial for the colonies in some cases, it often makes it more difficult for beekeepers to catch emerging problems and confidently assess the level of infection present in the colony by delaying the occurrence of colony level symptoms. Simply put, *N. ceranae*-infected individuals might be present in apparently healthy colonies without having any immediate consequences for the whole colony(Higes et al., 2008).

Pathophysiology is the study of changes in tissues that can either cause, result from, or are associated with a disease. As a tool, pathophysiology was used in early detection of some diseases in humans (Isidori et al., 2014; Tursi, Papa, & Danese, 2015). Linking physiological changes in the tissues with the accompanying disease can help elucidate the epidemiology of the disease (Martignoni, 1964). Pathophysiology was also previously proven useful in predicting CCD status of colonies (VanEngelsdorp et al., 2017). As previously explained, catching emerging diseases in managed colonies in early stages is often difficult for the beekeepers. When the *N. ceranae* infection is spread through the colony and starts having consequences on the colony level, it might be too late to initiate the treatment. Using pathophysiology as a tool, therefore, is a promising approach for better understanding and predicting the diseases presence in honey bee colonies. We think a pathophysiological approach might reveal significant associations between *N. ceranae* infection and the physiological symptoms observed in infected individuals/apiaries.

Previously, the outer sheath of ventriculus was thought to have a milky white or chalky appearance in *Nosema apis* infected bees (Hertig, 1923). Other pathophysiological studies on *N. ceranae* and *N. apis*, however, showed the ventriculus in infected individuals might not always appear chalky or milky white (Maiolino et al., 2014; Snow, 2016).

Another aspect of nosemosis is that the manifestation of the disease is often coincident with the presence of other stressors/pathogens (Hedtke, Jensen, Jensen, & Genersch, 2011; Christopher Mayack & Naug, 2009). Therefore, in our interpretation of previous literature, whether it is the cause or the consequence, *N. ceranae* infection might be considered a good indicator of a stressed colony. There is still, however, a need for work on gross organ level pathologies caused by *N. ceranae* infection. Such efforts may improve our understanding of this disease and provide applicable information for developing tools to assess the health state of the colonies before they become apparently symptomatic.

Here we set out to investigate the potential links, if any, between the bee physiology and *N. ceranae* infection. We wanted to compare physiological traits of bees coming or not coming from *N. ceranae* infected apiaries. Additionally, we wanted to assess if the apiary comparison results hold true when we group the bees by their infection status on an individual bee level. To explore these questions, we first dissected bees from *N. ceranae* positive and negative apiaries and scored their tissues. Following the dissections, we analyzed all the bees for the presence of *N. ceranae* spores. We then analyzed the data we collected with two different approaches; a comparison between *N. ceranae* positive and negative apiaries, and a comparison between individual bees that had and did not have spores in their gut tissues.

Materials and Methods

Sample Selection

Samples representing apiaries that had more than 1 million *N. ceranae* spores per bee were selected from NHBS 2017-2018 survey year (N=43 - High Nosema group). From the same database, samples that did not have any spores were also selected (N=623 - No-Nosema group). In order to minimize the possible effects of different environmental factors and high varroa loads, all the samples were grouped by the collection season and samples that had more than 3 mites per hundred bees were excluded (N=239). Finally, 5 from fall, 10 from spring, 22 from summer, in total 37 samples were selected from both high and no Nosema groups.

Necropsies and Data Collection

Bee samples from the National Honey Bee Survey were necropsied following the procedures described by Carreck N.L, et al.(Carreck et al., 2013) and scored to compare physiological differences between bees with and without the *Nosema* infection using the scoring criteria developed by vanEngelsdorp et al., 2017 and modified by Nearman, A., 2019 (Unpublished) (Table 1). We examined the tissues in the abdominal cavity, alimentary tract, sting apparatus, and hypopharyngeal glands for each bee. In total we compared nineteen different traits (Table 1). Ten bees were randomly selected from each apiary sample to observe the differences in their tissues. The excess ethanol was removed by placing these bees on a paper towel for several minutes. Then, the head was removed from the body and pinned on to a wax plate. The head was opened up using a scalpel by making lateral incisions along the inner

margins of the eyes, top of the head. Later, the remaining body of the bee was pinned onto the wax plate by the thorax and sting apparatus was pulled out using a forceps. Two lateral incisions were made in order to open up the abdominal cavity and the abdomen was detached from the thorax. Later, the head, abdomen and the sting apparatus was transferred onto another small petri dish filled with ethanol in order to examine the tissues under the microscope. Following the observations, all the bee parts were transferred into a small centrifuge tube filled with DI water. These parts were homogenized into a slurry using a pestle. An aliquot from this slurry was used to do a hemocytometer count for the *Nosema ceranae* spores.

Statistical Analysis

All statistical analyses were done using R programming language, version 4.0.4 (R Core Team, 2021). We employed Generalized Linear Mixed Models (GLMMs) and Cumulative Link Mixed Models (CLMM) to evaluate if the *N. ceranae* status of an apiary can be predicted by analyzing any of the scored individual pathophysiologies. We used apiary's *N. ceranae* status as the fixed effect while we used the binary scores of the pathophysiologies as the response variable. Bees' apiary IDs were used as the random effect in order to account for the variance caused by the spatial factors and management differences between apiaries. *Nosema* and control apiaries both had infected and non-infected individuals. We also compared bees that were and were not infected by using the individual bees' infection status as the fixed effect, and apiary IDs as the random effect.

We used the binary responses of each individual bee to calculate the prevalence of pathologies in bees coming or not coming from apiaries indicated as *N. ceranae* positive. We then run likelihood ratio tests to determine if any of the traits showed significant difference between the groups. All the p values from these tests were adjusted using Bonferroni correction method. We analyzed the traits with binary responses with logistic regression using the "glmer" function from lme4 package (Bates et al., 2020). Traits with ordinal responses were analyzed with cumulative logistic regression using the "clmm" function from ordinal package (Christensen, 2019). For two traits (rectal stones, sting gland coloration) some response levels were extremely rare that the GLMMs or CLMMs did not converge. We used chi-squared tests in order to determine whether these traits had significantly different prevalences in *Nosema* and control apiaries.

For traits that showed significant difference between groups, we calculated the odds ratios using "oddsratio" function from epitools package (Aragon, 2020).

Results

Comparing *N. ceranae* Apiaries with Control Apiaries

The spore counts within the samples that were indicated as having more than 1 million spores per bee are not normally distributed. Upon analyzing each bee for spores, we found *N. ceranae*-positive samples consist of many individuals without any spores and a smaller number of super-infected individuals (Figure 2). These seemingly non-infected bees, however, might be in the early stages of the disease, and

might show symptoms without having a detectable number of spores in their gut tissues. To account for this, we compared all bees from *N. ceranae* positive apiaries (including those without any spores) to bees from control apiaries (Table 2). In nineteen traits we tested, frequency of four traits showed significant difference in *Nosema* and control apiaries: venom sac coloration (Figure 3), black tissue (Figure 4), white nodules (Figure 5), sting gland melanization (Figure 6).

Bees from apiaries with high *N. ceranae* loads were ~3 times more likely to have discolored venom sacs (Figure 3b), melanized tissues (Figure 4b) and white nodules compared to the bees from control apiaries. They were also 15.7 times more likely to have melanized sting glands (Figure 6b) in comparison to the bees from apiaries with no *N. ceranae* detection.

Comparing bees with *N. ceranae* spores to bees with no spore detection.

In addition to the comparisons between bees from *N. ceranae* and control apiaries, we compared bees that had and did not have spores (Table 3). Four symptoms showed different prevalence in bees with and without spores: Venom sac debris (Figure 7), sting gland coloration (Figure 8), fecal matter consistency (Figure 9), and Malpighian tubule quantity (Figure 10).

Bees with *N. ceranae* spores were ~2 times more likely to have venom sac debris and discolored sting glands (Table 1; Score 1) as well as soft fecal matter (Table 1; Score 0). Bees with spores were also less likely to have hardened fecal matter (Table 1; Score 2). Bees without any spores were more likely to have a reduced quantity of Malpighian tubules.

Discussion

The results show there is a subset of physiological traits with different manifestation patterns between Nosema apiaries and the control apiaries. Our comparison demonstrated that bees coming from apiaries with *N. ceranae* exposure tend to have more discolored venom sacs, more melanized tissues, more white nodules, and more melanization on their sting glands. Two of these symptoms seem to overlap with age-related physiological changes (sting gland melanization, white nodules). Previous studies showed the prevalence of the *N. ceranae* infection is almost always higher in older forager bees when compared to younger house bees (Higes et al., 2008). It is also shown *N. ceranae* infection can artificially age bees causing precocious foraging behavior (Goblirsch et al., 2013). Sting gland melanosis is shown to be more prevalent in older forager bees (VanEngelsdorp et al., 2017). So, as expected, we saw increased odds of having melanized sting glands in bees from *Nosema* apiaries. White nodules, on the other hand, are more prevalent in younger house bees in comparison with foragers (VanEngelsdorp et al., 2017). Therefore, it was surprising to see white nodules being more prevalent in the Nosema apiaries. One possible explanation might be, in the colonies that are exposed to *N. ceranae* spores, a greater proportion of the older bees are dead due to the infection, so they are not being sampled. Thus, causing samples to disproportionately represent younger-aged house bees. The spore count distribution among the bees from Nosema apiaries is also consistent with this explanation (Figure 2). If we assume this explanation is correct (samples from Nosema apiaries are disproportionately representing younger bees), it seems like the higher odds of having black tissues and melanized sting glands in the

bees from Nosema apiaries are not necessarily related to actual older age. Suggesting they might be caused by an active immune response occurring in these bees.

Our results from comparing bees with and without spores did not overlap with the results from apiary comparisons. They had a different subset of traits that showed different manifestation patterns between bees with and without spores. Bees with spore loads had more discolored sting glands (Table 1; Score 1), more debris in their venom sacs, and less reduction in their Malpighian tubule quantities. They also had softer fecal matter (Table 1; Score 0) while bees without spores were more likely to have hardened fecal matter (Table 1; Score 2). *N. ceranae* infection is mainly targeting the intestines of the honey bees (Dussaubat et al., 2012). Furthermore, one of the symptoms of nosemosis is bees defecating on the walls of the hive box. Simply due to diarrhea caused by the infection. So, initially, it was not surprising to see the softer fecal matter in infected bees. In contrast, previous studies showed *N. ceranae* is not causing diarrhea like *N. apis* (Maiolino et al., 2014). One possible explanation might be the upregulation of a protein called corticotrophin-releasing-hormone (CRH)-binding protein in bees with *N. ceranae* infection (Dussaubat et al., 2012). This highly preserved protein between mammals and insects(Huising & Flik, 2005), mediates the gut response to stress in mammals (Stengel & Taché, 2009). Sting gland discoloration, however, was previously shown to decline with age (VanEngelsdorp et al., 2017). Therefore, seeing sting gland discoloration more prevalent in the bees with spores was contradictory to the assumption of *N. ceranae* infection is artificially aging the bees. On the other hand, bees with *N. ceranae* spores had more Malpighian tubules. Malpighian tubule quantities of the young bees are shown to be present in

reduced quantities, and as the bees age, it increases in quantity(VanEngelsdorp et al., 2017).

Overall, our results showed there are significant differences between our groups in both sets of analyses. This suggests that associations between *N. ceranae* and physiological changes in honey bees can be discovered through a pathophysiological approach. While we were able to discover these associations between *N. ceranae* exposure/infection and the pathology of the honey bees, it is also noteworthy that unraveling the entirety of the complex mechanism behind these symptoms was not within the scope of this retrospective cohort study. Future work can further investigate the symptoms identified here to provide more detailed explanations for these pathophysiological associations.

Tables and Figures

Variable/Characteristic	Categorization (score)	Description
Black tissue	Absent (0)	
	Present (1)	Spots of discolored muscle or connective tissue present in the abdominal cavity
White nodules	Absent (0)	
	Present (1)	Opaque white nodules can be found in or on abdominal tissues
Ventriculus size	Small (0)	0.5-1mm wide by 4-5mm long
	Medium (1)	1-1.2mm wide by 4.5-5.5mm long
	Large (2)	1.5-2.5mm wide by 5.5-6.5mm long
Ventriculus coloration	Light (0)	Sheath is >2/3 white, cream-colored, or translucent
	Medium (1)	Sheath mostly tan to brown
	Dark (2)	Sheath almost entirely brown or black
Pyloric scarring	Absent (0)	
	Present (1)	Dark band (scar) running partially or entirely around the perimeter of the pylorous region
Malpighian tubule color	Clear (0)	≥2/3 of the tubules translucent to cream colored
	Slight discoloration (1)	≥2/3 of the tubules tan to brown
	Severe discoloration (2)	≥2/3 of the tubules brown to black
Malpighian tubule quantity	Normal (0)	≥ ~50 tubules present
	Reduced (1)	< ~50 tubules present
Malpighian tubule iridescence	Normal (0)	
	Iridescent spots present (1)	Small iridescent spots seen along the Malpighian tubules
Fecal matter color	Light (0)	White or very light yellow
	Medium (1)	Orange, deep yellow, red, or light brown
	Dark (2)	Dark brown
Rectum distension	¼ full (0)	Fills 0-33% of the abdominal cavity
	½ full (1)	Fills 33-66% of the abdominal cavity
	Full (2)	Fills 66-100% of the abdominal cavity
Fecal matter consistency	Soft (0)	Ejects readily like a thick liquid from the rectum sac when probed
	Semi-hard (1)	Breaks apart in clumps when probed
	Hard (2)	Remains completely solid when probed
Enteroliths in rectum	Absent (0)	
	Present (1)	Hard concretions that look like small grains of rice present
Venom sac color	Translucent (0)	
	Discolored (1)	Any amount of discoloration visible
Venom sac debris	Absent (0)	
	Present (1)	Any sort of solid debris is present in the venom sac
Sting gland swelling	Normal (0)	Sting gland is of normal size
	Intermediate (1)	Sting gland is slightly larger than normal, and appears to have a second layer
	Very swollen (2)	Sting gland is extremely oversized
Sting gland tissue melanosis	Absent (0)	
	Present (1)	Sting gland contains areas of melanin
Sting gland color	Light (0)	Translucent to tan
	Medium (1)	Tan to brown
	Dark (2)	Brown to dark brown
Hypopharyngeal glands	Developed (0)	Glands are round and fill entirety of head cavity above brain
	Atrophied (1)	Glands are flattened and/or do not fill entirety of head cavity above brain
Fat body content	Normal (0)	Fat body opaque
	Reduced (1)	Fat body almost translucent

Table 1: *Scoring criterion used to evaluate honey bee pathologies. Adapted from vanEngelsdorp et al. [15] with additional variables of the hypopharyngeal glands and fat body content (Nearman, A., 2019(Unpublished)*

***Table 2**: Frequencies of pathophysiological traits in bees from N. ceranae positive versus control apiaries. Test results are from likelihood ratio tests or, if marked with '§', chi-squared tests. '*' indicates traits that were significantly different at α = 0.05. p values were adjusted using Bonferroni correction method.*

	Bees from *Nosema c.* apiaries				**Bees from control apiaries**				**Likelihood ratio test (*Nosema c. positive* vs. *Nosema c. negative*)**	
Variable	**N**	**Score 0 (%)**	**Score 1 (%)**	**Score 2 (%)**	**N**	**Score 0 (%)**	**Score 1 (%)**	**Score 2 (%)**	**χ2**	***P* (adjusted)**
Hypopharangeal Glands	370	74.32	25.68	NA	370	72.7	27.3	NA	0.14	1
Venom Sac Debris	370	53.78	46.22	NA	370	67.84	32.16	NA	6.31	0.12
Venom Sac Coloration	370	79.46	20.54	NA	370	92.7	7.3	NA	10.83	0.01*
Sting Gland Swelling	370	50	37.57	12.43	370	55.14	25.68	19.18	0.02	1
Sting Gland Melanosis	370	91.62	8.38	NA	370	99.46	0.54	NA	4.03	0.004*
Sting Gland Coloration	370	90.54	9.46	0	370	94.59	5.41	0	3.85§	0.05
Black Tissue	370	72.43	27.57	NA	370	90	10	NA	17.82	<0.001*
Rectum Distention	370	28.38	45.95	25.67	370	29.19	42.16	28.65	0.1	1
Fecal Matter Color	370	23.78	73.51	2.71	370	27.03	71.62	1.35	0.97	1
Fecal Matter Consistency	370	62.43	25.68	11.89	370	62.43	27.3	10.27	0.02	1
Rectal Stones	370	95.95	4.05	NA	370	96.76	3.24	NA	0.28	0.59
Ventriculus Size	370	32.43	40.54	27.03	370	15.68	56.76	27.56	2.64	0.73
Ventriculus Coloration	370	81.08	16.49	2.43	370	91.08	8.11	0.81	7.13	0.05
Pyloric Scarring	370	64.05	35.95	NA	370	73.24	26.76	NA	5.6	0.18
Malpighian Tubule Color	370	80.54	19.19	0.27	370	87.57	12.43	0	2.25	0.93
Malpighian Tubule Quantity	370	44.32	55.68	NA	370	30.54	69.46	NA	6.35	0.12
Malpighian Tubule Iridescence	370	97.3	2.7	NA	370	96.22	3.78	NA	0.06	1
Fat body reduction	370	25.95	74.05	NA	370	26.49	73.51	NA	0.11	1
White Nodules	370	74.59	25.41	NA	370	90.81	9.19	NA	12.53	0.004*

***Table 3**: Frequencies of pathophysiological traits in bees with and without N. ceranae spores. Test results are from likelihood ratio tests or, if marked with '§', chi-squared tests. '*' indicates traits that were significantly different at α = 0.05. p values were adjusted using Bonferroni correction method.*

	Bees with *Nosema c.* spores				**Bees without *Nosema c.* spores**				**Likelihood ratio test (*Nosema c. positive* vs. *Nosema c. negative*)**	
Variable	**N**	**Score 0 (%)**	**Score 1 (%)**	**Score 2 (%)**	**N**	**Score 0 (%)**	**Score 1 (%)**	**Score 2 (%)**	**χ2**	***P* (adjusted)**
Hypopharangeal Glands	90	61.11	38.89	NA	650	75.23	24.77	NA	6.28	0.12
Venom Sac Debris	90	43.33	56.67	NA	650	63.23	36.77	NA	9.21	0.02*
Venom Sac Coloration	90	68.89	31.11	NA	650	88.46	11.54	NA	6.11	0.13
Sting Gland Swelling	90	43.33	42.22	14.45	650	53.85	30.15	16	2.17	0.98
Sting Gland Melanosis	90	88.89	11.11	NA	650	96.46	3.54	NA	3.84	0.49
Sting Gland Coloration	90	86.67	13.33	0	650	93.38	6.62	0	4.26§	0.049*
Black Tissue	90	81.11	18.89	NA	650	81.23	18.77	NA	0.84	1
Rectum Distention	90	37.78	47.78	14.44	650	27.54	43.54	28.92	7.09	0.054
Fecal Matter Color	90	30	68.89	1.11	650	24.77	73.08	2.15	1.76	1
Fecal Matter Consistency	90	78.89	18.89	2.22	650	60.15	27.54	12.31	11.18	0.006*
Rectal Stones	90	100	0	NA	650	95.85	4.15	NA	2.78§	0.95
Ventriculus Size	90	33.33	26.67	40	650	22.77	51.69	25.54	6.48	0.08
Ventriculus Coloration	90	82.22	17.78	0	650	86.62	11.54	1.84	0.15	1
Pyloric Scarring	90	58.89	41.11	NA	650	70	30	NA	3.46	0.62
Malpighian Tubule Color	90	77.78	22.22	0	650	84.92	14.92	0.16	0.12	1
Malpighian Tubule Quantity	90	60	40	NA	650	34.31	65.69	NA	14.04	0.002*
Malpighian Tubule Iridescence	90	95.56	4.44	NA	650	96.92	3.08	NA	0.51	1
Fat body reduction	90	26.67	73.33	NA	650	26.15	73.85	NA	0.27	1
White Nodules	90	78.89	21.11	NA	650	83.23	16.77	NA	0.03	1

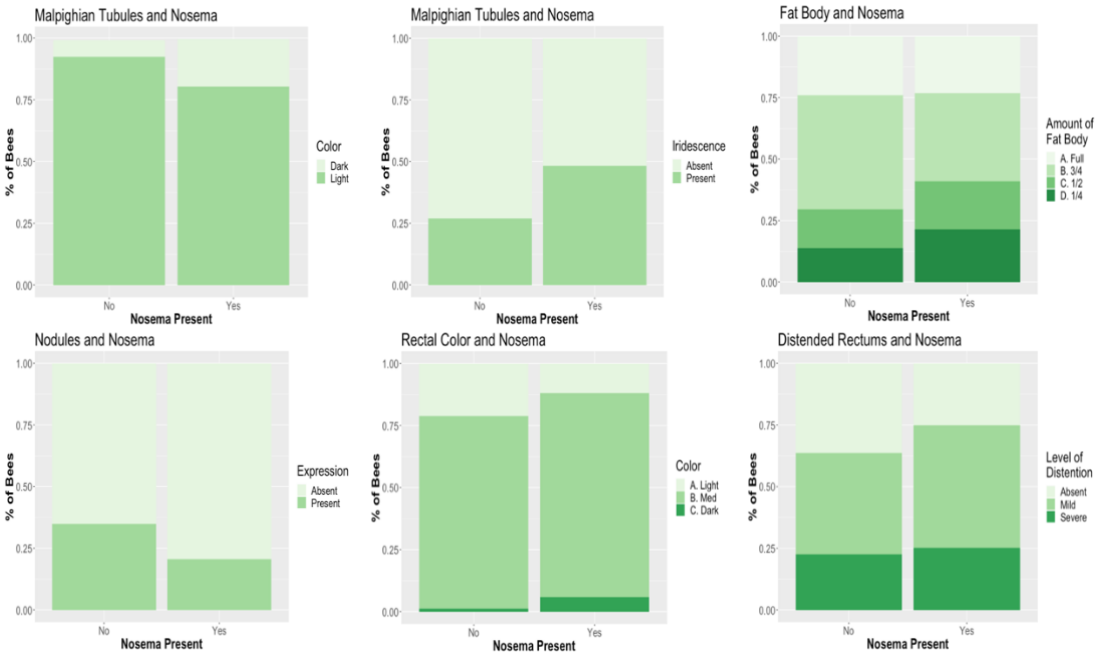

Figure 2: *Preliminary results showing associations between Nosema infection and several physiological traits (Nearman, A. -Unpublished).*

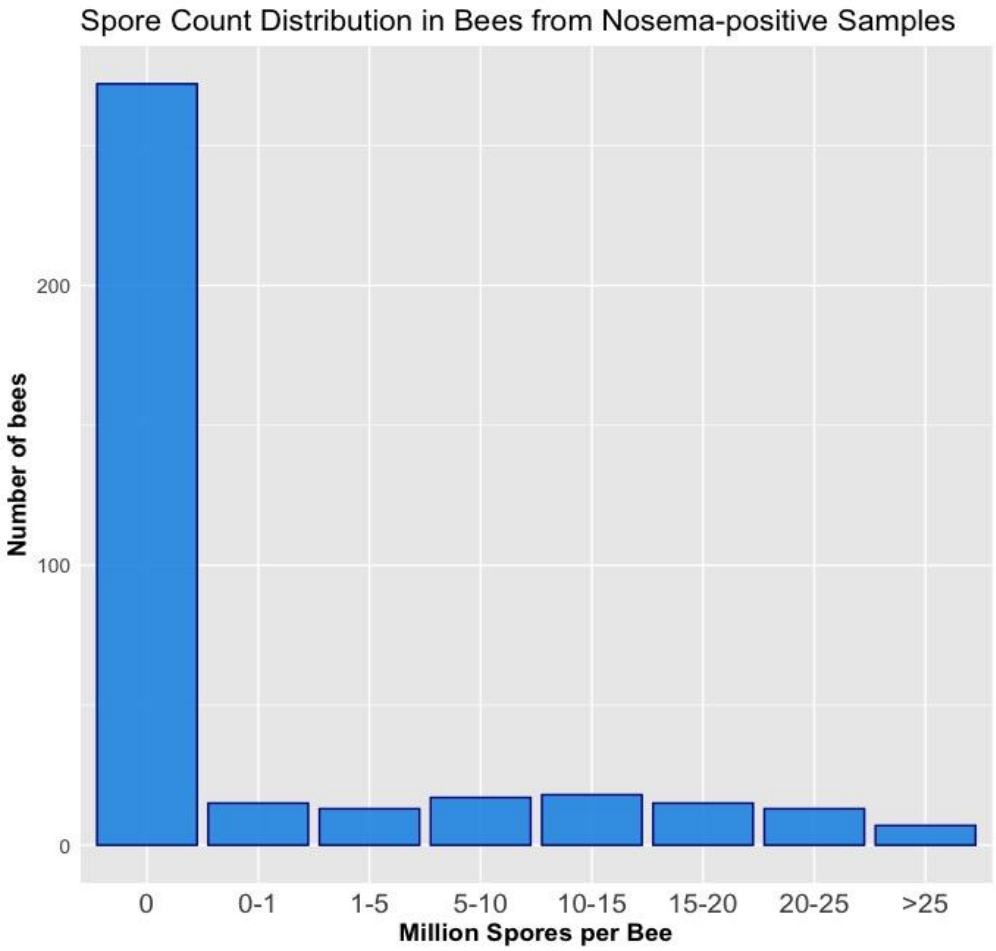

***Figure 2**: Spore count distribution in samples from Nosema positive apiaries. (N=370)*

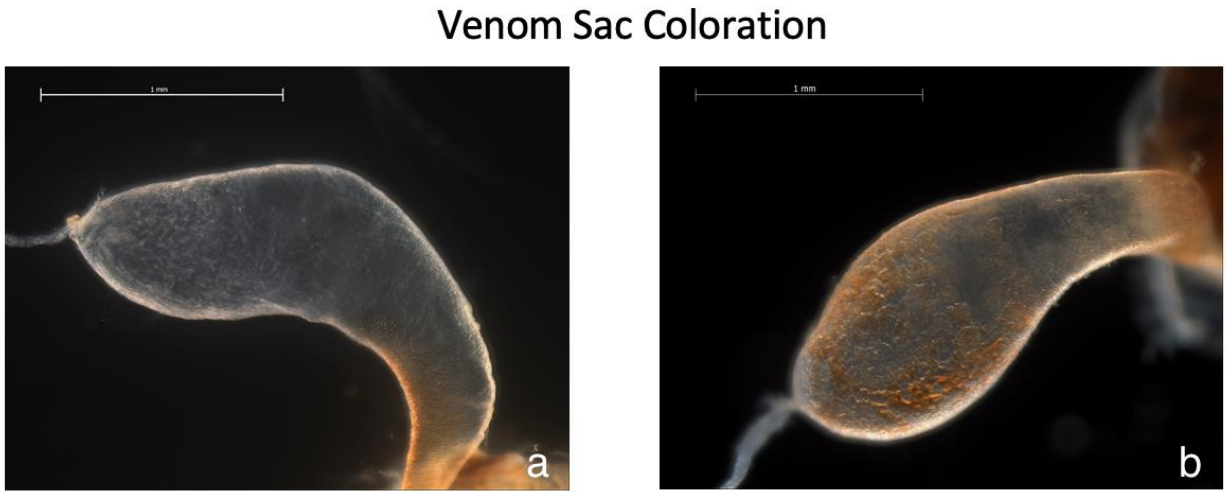

Figure 3*: (a)Translucent venom sac tissues and (b)discolored venom sac tissues. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

Black Tissue

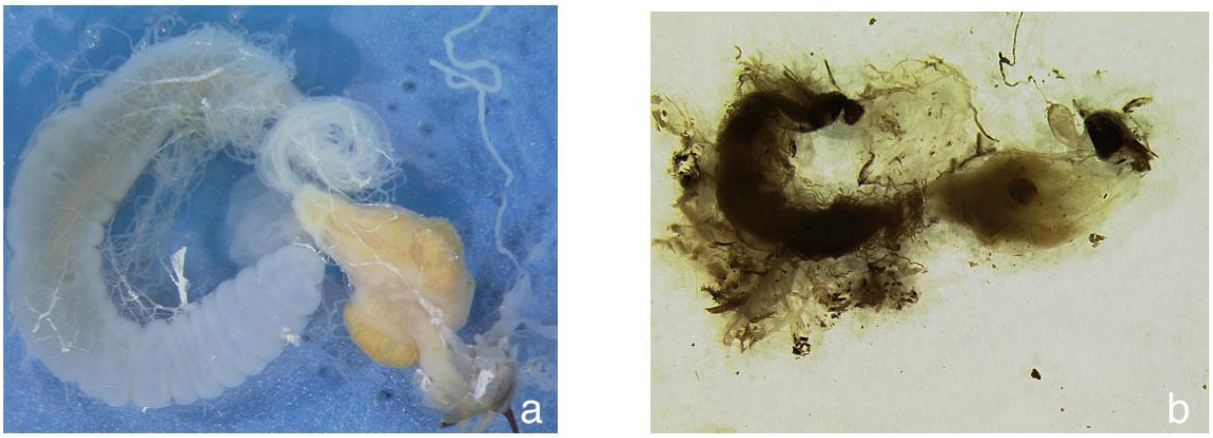

Figure 4*: (a)Healthy gut tissues and (b)melanized gut tissues. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

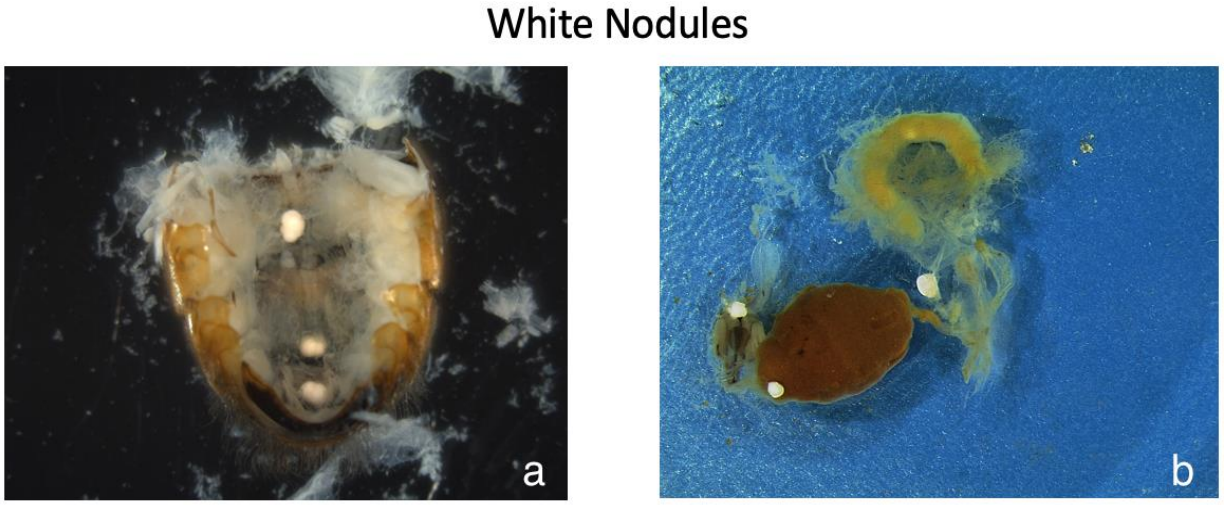

Figure 5: *(a)White nodules on tergites and (b)white nodules on gut tissues. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

Sting Gland Melanization

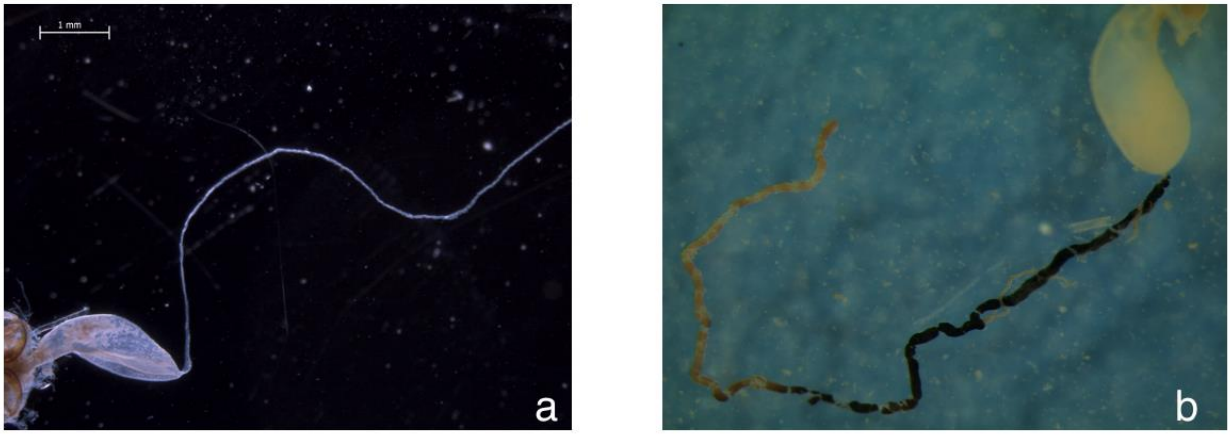

Figure 6*: (a)Clear sting gland and (b)melanized sting gland. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

Venom Sac Debris

Figure 7*: (a)Clear venom sac and (b)venom sac with debris. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

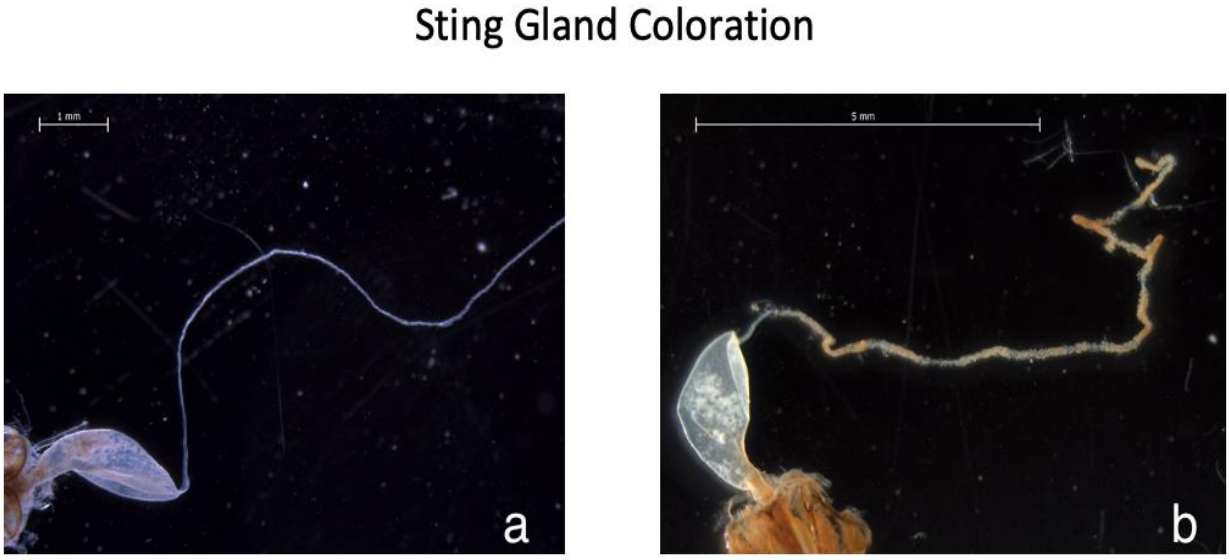

Figure 8*: (a)Clear sting gland and (b)discolored sting gland. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

Figure 9*: (a)Soft fecal matter, (b)semi-hard fecal matter, and (c)hardened fecal matter. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

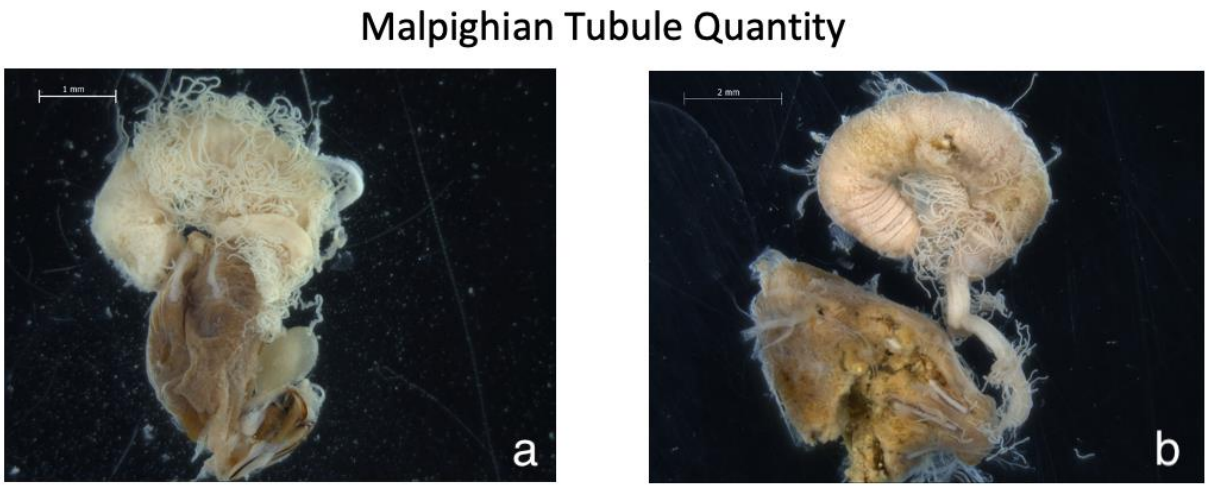

Figure 10*: (a)Normal Malpighian tubule quantity and (b)reduced Malpighian tubule quantity. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

Chapter 2: Exposure to *Nosema ceranae* Advances the Onset of Certain Age-Related Pathophysiologies in Worker Honey Bees.

Introduction

Nosema ceranae is known to cause reduced lifespan, precocious foraging, altered energy metabolism, and suppressed immune systems in honey bees (Higes et al., 2008; Christopher Mayack & Naug, 2009; Woyciechowski & Moroń, 2009).

Honey bees are eusocial insects displaying complicated patterns of temporal polyethism(Huang & Robinson, 1996). *N. ceranae* is thought to have adverse effects on colony health by interfering with the mechanisms regulating the temporal polyethism(Woyciechowski & Moroń, 2009). Although these behavioral changes are observed in *N. ceranae* infected individuals, underlying pathophysiologies needs further exploration.

N. apis is well studied in terms of its epidemiology. On the other hand, there is still a need for more work to elucidate the consequences of the *N. ceranae* infection for the bees from a pathophysiological perspective. Our observational study found associations between pathologies that were more prevalent in bees coming from *Nosema* infected apiaries. Finding associations between exposures and gross pathologies using observational studies are suggestive but by no means definitive. Therefore, we wanted to see if there is any causative correlation between the pathophysiological traits and the *N. ceranae* infection through cage experiments.

We also wanted to see the progression of the infection, and possible associations between the infection and the pathologies.

Here we set out to investigate how the risks of having these pathologies change with exposure to *N. ceranae* spores and how these symptoms are developing over time as the disease progresses in the bee body. We wanted to compare the physiological traits of bees that were exposed and not exposed to *N. ceranae* spores. In addition, we wanted to see how the prevalence of these symptoms are changing over time in bees that were and were not exposed. We devised a cage experiment to explore these questions. We inoculated a group of bees with *N. ceranae* spores and collected samples from cages at different timepoints. We first analyzed the differences between the frequencies of specific pathologies in exposed and unexposed bees. Some of these symptoms are age/task dependent, and they might not even show up until later in the bee's life. For this reason, we wanted to avoid grouping all the control and treatment bees from all the age groups together. We wanted to see if there are differences present in bees that lived in the exposed versus unexposed cages at day twenty. Therefore, we compared the bees we collected from control and treatment cages on day twenty. We then compared exposed and unexposed bees in terms of pathologies occurring in their bodies after we grouped the samples based on the collection day. This allowed us to see how these changes are developing over time in the bee body.

Materials and Methods

Cultivating Spores

In order to initiate the experimental studies, we needed a sufficient supply of *N. ceranae* spores. Therefore, cultivating spores in the lab settings was essential. To do this, worker bees from colonies with known *N. spp.* infections were located at the Central Maryland Research and Education Center (CMREC) – Beltsville Facility (City state) and harvested. Collected bees were put into cages (16 oz. disposable solo cups). 20 bees per cage were randomly selected and analyzed for presence of *N. ceranae* spores using light microscopy. Once the *N. ceranae* spores were observed in the sample, bees from the source cage were used to prepare a spore inoculum following the procedures described by Fries et al., 2013(Ingemar Fries et al., 2013). *N. ceranae* spores are losing their viability significantly when stored over 24 hours(Fenoy, Rueda, Higes, Martín-Hernández, & Del Aguila, 2009). We, therefore, needed a constant and fresh supply of spores. In order to cultivate more spores in laboratory, two frames of capped brood were collected from an apparently disease-free colony. They were placed in frame cages and kept in the incubator. After 24 hours of incubation, newly emerged bees were collected and put into cages with feeders. From these two brood frames we were able to fill eight cages with 480 bees (60 bees each). We then randomly selected four of these cages to be fed *N. ceranae* inoculum containing $99x10^4$ spores per ml, equivalent to $33x10^3$ spores per bee. $33x10^3$ spores per bee is sufficient to infect caged bees in a group feeding setting (Ingemar Fries et al., 2013; Pettis, Vanengelsdorp, Johnson, & Dively, 2012; Webster, 1994). These cages later used to produce spore inoculums for the treatment

group of the experiment. The remaining four cages received 50% sucrose solution and were used to prepare blank inoculums for the control group of the experiment. To ensure the bees are infected, they were harvested from inoculated cups 12 days post-inoculation (Ingemar Fries et al., 2013). Presence of *N. ceranae* was confirmed using qPCR methods(Forsgren & Fries, 2010). They were, then, used to prepare inoculums for the cage experiments following the procedures described by Fries et al (Ingemar Fries et al., 2013). Equal number of bees from the 4 non-infected cups were used to prepare blank inoculums in order to inoculate the control group later in the cage experiments.

Cage Experiments

Colonies at CMREC, with no known *N. spp.* infection were identified. Frames of capped brood were collected from 4 different colonies and kept in the incubator at 34.5 °C, 70% relative humidity for optimal brood development (Human et al., 2013). Newly emerged bees were collected (< 24 hours old) and placed into 16 oz. disposable solo cups with feeders. In total, 18 cages were prepared, with each cage containing 60 bees. Cages were randomly assigned to equally sized control and inoculum treated groups. On the first day of the experiment, cages of bees assigned to inoculum treatment were group fed 1mL of 50% sucrose solution containing $198x10^4$ spores ($33x10^3$ spores X 60) over the course of 24 hours. Bees in the control cages were fed a spore-free 50% sucrose solution prepared by using the "blank inoculum" prepared earlier. Following the initial inoculation period, the first 24 hours, all bees had access to 50% sucrose solution and Megabee (prepared using the instructions on the label) *ad libitum* for the for the remaining duration of the trial. Sucrose solution

feeders were changed daily. Megabee feeders were changed every other day. Dead bees from all the cages were collected and recorded daily to later analyze the cumulative mortality rates of control and treatment groups.

On days 1,3,7,12,15, randomly selected 5 bees from each cage and on day 20, all the alive bees were collected and preserved in 70% ethanol. To avoid adding any bias while dissecting and scoring the samples, all the vials were labeled with randomly generated six-digit numbers. To later unblind the results, the original identifiers (and the linked six-digit numbers) of the samples were recorded in a separate document. Later, dissections were performed individually to assess the physiological differences between infected and non-infected bees. After the dissections were done and the data was entered, the unique six-digit numbers were deciphered, and samples were matched with their original identifiers.

Necropsies and Data Collection

Samples collected from cage studies were preserved in 70% ethanol. Later, necropsies were carried out using procedures described by Carreck N.L, et al.(Carreck et al., 2013) In addition to previously developed 17 specific pathophysiological differences criterion by vanEngelsdorp et al., 2017, we scored fat body quantity and the development of hypopharyngeal glands (Nearman, A. Unpublished 2019) to link any possible physiological differences seen in these tissues with the presence of *Nosema ceranae*. We examined the tissues in the abdominal cavity, alimentary tract, sting apparatus, and hypopharyngeal glands for each bee. In total we compared nineteen different traits (Table 1). All of the five bees from each sample vial were removed to observe the differences in their tissues. The excess

ethanol was removed by placing these bees on a paper towel for several minutes. Then, the head was removed from the body and pinned on to a wax plate. The head was opened up using a scalpel by making lateral incisions along the inner margins of the eyes, top of the head. Later, the remaining body of the bee was pinned onto the wax plate by the thorax and sting apparatus was pulled out using a forceps. Two lateral incisions were made in order to open up the abdominal cavity and the abdomen was detached from the thorax. Later, the head, abdomen and the sting apparatus was transferred onto another small petri dish filled with ethanol in order to examine the tissues under the microscope. Following the observations, all the bee parts were transferred into a small centrifuge tube filled with DI water. These parts were homogenized into a slurry using a pestle. An aliquot from this slurry was used to do a hemocytometer count for the *Nosema ceranae* spores.

Statistical Analysis

All statistical analyses were done using R programming language, version 4.0.4[35].

Comparisons Between Control and Treatment Bees on Day Twenty

We employed Generalized Linear Mixed Models (GLMMs) and Cumulative Link Mixed Models (CLMM) to evaluate if the exposure to *N. ceranae* spores can cause significant differences in the prevalence of pathophysiologies between control and treatment groups on bees that survived until day twenty. We used the exposure group (control/treatment) as the fixed effect while we used the binary scores of the

pathophysiologies as the response variable. We compared different models against one another using source colonies, cage IDs, and both of these variables as the random effect. We found using cage IDs as a random effect was resulting in better fitting models. All meaningful models were tested against the null models.

We used the binary responses of each individual bee to calculate the prevalence of pathologies in bees exposed or not exposed to *N. ceranae* spores. We then run likelihood ratio tests to determine if any of the traits showed significant difference between the control and treatment groups. We used same method for comparisons of multiple traits. We, therefore, adjusted p values from these tests using Bonferroni correction method. We analyzed the traits with binary responses with logistic regression using the "glmer" function from lme4 package (Bates et al., 2020). Traits with ordinal responses were analyzed with cumulative logistic regression using the "clmm" function from ordinal package(Christensen, 2019).

For traits that showed significant difference between groups, we calculated the relative risk using "riskratio" function from epitools package(Aragon, 2020).

Comparing Control and Treatment Bees Grouped by the Time Variable

We grouped samples by the age of the bees and compared prevalence of the pathophysiological traits in control and treatment groups within each age group. We had six groups: day1, day3, day7, day12, day15, day20. Samples for all the days were of equal size (control N=45, treatment N=45), except for the day 20. Day 20 was the

last day of the trial. We, therefore, collected all the remaining bees (N=173 for treatment group, N=279 for control group).

Survival Probability Analysis

Kaplan-Meier curves were generated using "survfit" from survival package(Therneau, 2020) and "ggsurvplot" function from survminer package(Kassambara, Kosinski, & Biecek, 2021).

Results

Comparisons Between Bees from Control and Treatment Groups

As expected, *N. ceranae* exposure significantly reduced the survival probability of bees surviving at least 20 days (only ~50% of exposed bees survived compared to, ~80% for control bees) (Figure 31). As previous work showed, some of these traits are age related. They, therefore, are not showing up in our samples until in the later days of the trial. This could have resulted in skewed results for the said traits. For this reason, instead of grouping all the bees from control and treatment groups, we limited our comparisons to the bees that were sampled on day twenty. We then compared the bees that survived until day twenty from the group that was exposed to *N. ceranae* spores to see if we can link any of the pathophysiologies to the exposure to spores (Table 4). Frequencies of six traits showed significant difference between control and treatment groups on day twenty: Hypopharyngeal glands (Figure 12), venom sac coloration (Figure 3), sting gland swelling (Figure 11), and pyloric scarring (Figure 13), Malpighian tubule quantity and white nodules.

The exposure to *N. ceranae* spores increased the risk of having atrophied hypopharyngeal glands on day twenty 1.3 ($p<.001$). Exposed bees were also ~6 times more likely to have scarred pyloric valves compared to the unexposed bees ($p<.001$). The risk of having discolored venom sacs was increased by ~3 in exposed bees ($p<.001$). Sting glands of the exposed bees were more likely to be normal ($p<.001$) (Table 1; Score 0) and less likely to be swollen ($p<.001$) (Table 1; Score 2) on day twenty. We also saw an increased risk of having reduced quantities of Malpighian tubules in exposed bees compared to unexposed bees ($p = .0092$). Exposure to *N. ceranae* spores also reduced the relative risk of having white nodules ($p<.001$) on day twenty.

Time Series

To better understand the progression of *N. ceranae* infection and how the associated pathologies are developing over time in the bee body, we grouped the samples based on their sampling dates. We had 6 groups (days 1,3,7,12,15,20). Frequencies of ten traits were different between control and treatment groups on certain days: atrophied hypopharyngeal glands; day 12 (X^2 (1, N = 90) = 10.04, p = .0015), day 15 (X^2 (1, N = 90) = 12.66, p < .001), day 20 (X^2 (1, N = 452) = 26.83, p < .001) (Figure 14), pyloric scarring; day 15 (X^2 (1, N = 90) = 5.51, p = .01), day 20 (X^2 (1, N = 452) = 68.87, p < .001) (Figure 15), reduced Malpighian tubule quantity; day 3 (X^2 (1, N = 90) = 7.25, p = .007), day 20 (X^2 (1, N = 452) = 18.03, p < .001) (Figure 16), fat body reduction; day 7 (X^2 (1, N = 90) = 3.97, p = .046), day 12 (X^2 (1, N = 90) = 6.51, p = .01) (Figure 17), discolored venom sac; day 12 (X^2 (1, N = 90) = 4.14, p = .041), day 15(X^2 (1, N = 90) = 6.15, p = .01), day 20 (X^2 (1, N = 452)

= 17.55, $p < .001$) (Figure 18), normal sting glands; day 20 (X^2 (1, N = 452) = 85.98, p <.001) (Table 1; Score 0, Figure 19), intermediately swollen sting glands; day 20 (X^2 (1, N = 452) = 4.66, p = .03) (Table1; Score 1, Figure 10) very swollen sting glands; day 12 (X^2 (1, N = 90) = 4.38, p = .036), day 15 (X^2 (1, N = 90) = 10.41, p = .001), day 20 (X^2 (1, N = 452) = 57.83, $p < .001$) (Table 1; Score 2, Figure 19), medium colored ventriculi; day 15 (X^2 (1, N = 90) = 5.75, p = .016) (Table 1; Score 1, Figure 20b), light colored fecal matter; day 20 (X^2 (1, N = 452) = 14.69, $p <$ .001), medium colored fecal matter; day 20 (X^2 (1, N = 452) = 13.75, $p < .001$), light colored sting glands; day 15 (X^2 (1, N = 90) = 4.78, p = .028), medium colored sting glands; day 15 (X^2 (1, N = 90) = 5.86, p = .015), white nodules; day 12 (X^2 (1, N = 90) = 5.20, p = .02).

On day one, there were no significant differences observed for any of the traits. On day three, the only trait that showed the significant difference was Malpighian tubule quantities; bees from the treatment group were less likely to have reduced quantities of the tubules. On day twelve, bees from the treatment group had more atrophied hypopharyngeal glands and fat body reduction as well as venom sac coloration. Treatment bees also had less swollen sting glands (Table 1; Score 2) and white nodules.

On day fifteen, bees that were exposed to *N. ceranae* had more atrophied hypopharyngeal glands, scar tissue on their pyloric valves, light-colored sting glands and discolored venom sacs. They also had less medium-colored sting glands, medium colored ventriculi and severely swollen sting glands on day fifteen.

Day twenty was the day with the greatest number of significantly different traits. On day twenty, while intermediately and severely swollen sting glands were less prevalent, normal sting glands were more prevalent in bees from the treatment group. A greater proportion of the treatment bees also had pyloric scarring, Malpighian tubule quantity reduction, medium colored fecal matter discolored venom sacs and atrophied hypopharyngeal glands compared to control bees. Light colored fecal matter was less prevalent in bees from treatment group on day twenty.

Discussion

Comparisons Between Bees from Control and Treatment Groups

The results showed the exposure to *N. ceranae* spores can be linked to pathophysiological changes in the bee body. Our analysis demonstrated bees that were from the treatment group were more likely to have atrophied hypopharyngeal glands, discolored venom sacs, pyloric scarring and normal sting glands while they were less likely to have swollen sting glands. All four of these traits are overlapping with age related physiological changes found by previous work (Ahmad, Khan, Khan, & Li, 2021; Huang & Robinson, 1996; VanEngelsdorp et al., 2017).

Our results demonstrated atrophied hypopharyngeal glands were more prevalent in bees that were exposed to *N. ceranae* spores. Previous studies has shown the physiology of this tissue is highly dependent on the age and the cast of the bee (Ahmad et al., 2021; Huang & Robinson, 1996). *N. ceranae* infection is shown to promote aging, causing precocious foraging behavior (Goblirsch et al., 2013) and

hypopharyngeal glands are degenerating at the onset of foraging behavior (Huang & Robinson, 1996) therefore the increased odds of having atrophied hypopharyngeal glands in bees from treatment group was not unexpected. Nevertheless, the exact pathway of the interaction between *N. ceranae* and these glands begs further investigation.

Based on our results, another symptom with different manifestation patterns between control and treatment was sting gland swelling. According to previous studies, swollen sting glands are more prevalent in younger house bees, while normal sting glands are more frequent in older forager bees (VanEngelsdorp et al., 2017). In our results, we saw the bees that were exposed to *N. ceranae* spores were more likely to have sting glands that are not swollen. Under the assumption of *N. ceranae* is artificially aging bees, this finding is in compliance with the literature.

Our analysis showed the bees that were exposed to *N. ceranae* spores had higher odds of having a scarred tissue on their pyloric valve. Previous work demonstrated the prevalence of pyloric scarring was higher in older bees when compared to younger bees (VanEngelsdorp et al., 2017). Still, it was interesting to see it being more prevalent in bees that were exposed to *N. ceranae* spores in our experiment. This is simply because pyloric scarring is caused by a completely different pathogen, the bacterium *Frischella perrara* (Engel, Bartlett, & Moran, 2015). One possible explanation might be the suppressed immune system in treatment bees caused by the *N. ceranae* infection. Pyloric scarring is also an age related trait with a prevalence increasing over time (VanEngelsdorp et al., 2017). Therefore, the increased prevalence in treatment bees might be caused by the artificial aging of the

bees due to *Nosema* infection. Another explanation would be the possibility that our *N. ceranae* inoculation introduced the pathogen into the treatment group. Although, we prepared a blank inoculum from the bees coming from the same source colony as the bees we prepared the *Nosema* inoculum to avoid introducing other pathogens to only one group but not the other. In addition, while in smaller proportions, bees with pyloric scarring were also present in control group. We, therefore, think this was probably caused by a combination of our first two explanations. In any case, the exact link between *N. ceranae* infection and pyloric scarring requires further studying.

Our results also showed bees from the treatment group were more likely to have discolored venom sacs. This symptom was also significantly more prevalent in *Nosema* exposed bees in the apiary level analysis of our retrospective cohort study. While *N. ceranae* mainly targets the midgut epithelium of the honey bees (Christopher Mayack & Naug, 2009), some studies detected *N. ceranae* in other tissues in the bee body including salivary glands, fat body, hypopharyngeal glands, ovaries(Chen et al., 2009; Copley & Jabaji, 2012; Traver & Fell, 2012). Copley and Jabaji (2012) suggested the ovipositor, a channel used by both venom sacs and ovaries, might lead the presence of *Nosema* species in the venom sac tissue. There is, however, not enough evidence to support this theory.

We also saw an increased prevalence of white nodules in control bees. We know white nodules are indicators of an active immune response occurring in the body of the bees(VanEngelsdorp et al., 2017)

Time Series

We wanted to see how these symptoms are developed over time as the disease progresses in the bee body.

We found the frequency of atrophied hypopharyngeal glands were increasing with age and are diverging between control and treatment on day twelve. The frequencies stayed significantly different through day twenty (Figure 14). As we know these glands are mainly used for food secretion and more developed in nurse bees while atrophied in older forager bees(Huang & Robinson, 1996). Bees from control group also had increased amounts of atrophied hypopharyngeal glands, but the prevalence increased at a much smaller pace in this group compared to the prevalence in treatment group. A possible explanation for this pattern is *N. cerenae* infection is advancing the onset of hypopharyngeal gland degeneration causing a greater proportion of the treatment bees to have atrophied glands starting from day twelve through day twenty.

We also found the frequencies of very swollen sting glands were getting smaller over time, eventually diverging between control and treatment on day twelve, staying significantly less prevalent in treatment bees through day twenty. Sting gland swelling is an age related trait decreasing with age(VanEngelsdorp et al., 2017). In compliant with the previous studies, we saw a downward trend in the prevalence of this trait in control bees as well. The rate of the frequency decline, on the other hand, was not as fast as the decline rate of the treatment bees. Thus, in our interpretation of the data, much like the hypopharyngeal glands, the onset of sting gland degeneration might also be advanced by the presence of *N. ceranae*.

Malpighian tubules were present in larger quantities in the treatment bees on day three. While primarily targeting midgut tissues, *N. ceranae* is present in other tissues, including Malpighian tubules, as well (Chen et al., 2009). If we assume the presence of *Nosema* on a tissue is causing some pathologies to show up on the tissue, it was not surprising to see a change in the tubule quantity in the groups that were exposed to *N. ceranae.* In addition, this difference between control and treatment might suggest artificial aging in *N. ceranae* infected bees might start showing symptoms on tissues as early as the third day of the infection. In contrast, we saw the Malpighian tubule quantity reduction was higher in treatment bees on day twenty. This was surprising to see considering the tubule quantity is shown to increase with age (VanEngelsdorp et al., 2017). A possible explanation to this would be, if we assume that increased number of Malpighian tubules is a trait linked to bee mortality, the bees that had increased numbers of tubules did not survive until day twenty in the treatment group. Thus, they are not being sampled on day twenty, introducing survivorship bias into the day twenty analyses. If this assumption is correct (Malpighian tubule quantity is a trait linked to bee mortality), it would mean the difference we saw on day twenty was because bees with less tubules were disproportionately represented in day twenty. We think the relationship between *N. ceranae* spore exposure and Malpighian tubule quantity demands further studying.

The chalky white coloration on ventriculus sheath was previously shown to be correlated with *Nosema* infection (Hertig, 1923). Other studies later claimed that it was not always the case (Maiolino et al., 2014)(Snow, 2016). We found on day fifteen, the prevalence of medium-colored ventriculi (Table 1; Score 1) in treatment

bees was significantly smaller than that of control bees. Also, on day fifteen, light-colored ventriculi were more prevalent in treatment bees while medium-colored ventriculi were less prevalent.

Fat body was the symptom that we found most interesting. We saw on day seven, the prevalence of fat body reduction was higher in control bees. On day twelve, however, the situation was inversed. We saw increased prevalence of fat body reduction in treatment bees on day twelve. This is interesting especially considering the Kaplan Meier curves for control and treatment was significantly diverged between control and treatment groups on day twelve. In addition, we know Vitellogenin(Vg), a glicolipoprotein crucial for the temporal polyethism in honeybees(Münch & Amdam, 2010), is synthesized in fat body (Byrne, Gruber, & Ab, 1989). The lack of Vg is shown to cause extremely precocious foraging behavior in honey bees(Marco Antonio et al., 2008). In light of these findings, this reduction of fat body in our treatment group on day twelve might explain the precocious foraging behavior in *N. ceranae* infected bees.

We did not find any trait that had statistically different frequencies between treatment and control for day one. Although not significantly different, we thought it was still noteworthy on day one, the increased prevalence of sting gland melanosis seemed to be implying a strong immune response in the treatment bees (Figure 22).

Tables and Figures

***Table 4**: Frequencies of pathophysiological traits in bees that were exposed to N. ceranae spores versus unexposed bees on day twenty. Test results are from likelihood ratio tests or, if marked with '§', chi-squared tests. '*' indicates traits that were significantly different at α = 0.05. p values were adjusted using Bonferroni correction method.*

	Bees from treatment group on day 20				Bees from control group on day 20				Likelihood ratio test (control vs treatment day 20)	
Variable	**N**	**Score 0 (%)**	**Score 1 (%)**	**Score 2 (%)**	**N**	**Score 0 (%)**	**Score 1 (%)**	**Score 2 (%)**	**χ2**	***P* (adjusted)**
Hypopharangeal Glands	173	16.18	83.82	NA	279	39.78	60.22	NA	12.39	0.004*
Venom Sac Debris	173	71.68	28.32	NA	279	69.89	30.11	NA	0.09§	0.77
Venom Sac Coloration	173	84.97	15.03	NA	279	96.42	3.58	NA	13.54	0.002*
Sting Gland Swelling	173	68.21	21.39	10.4	279	23.66	31.18	45.16	21.98	<0.001*
Sting Gland Melanosis	173	99.42	0.58	NA	279	100	0	NA	0.06§	0.81
Sting Gland Coloration	173	93.06	6.94	0	279	92.83	7.17	0	0.0016§	0.97
Black Tissue	173	98.27	1.73	NA	279	96.77	3.23	NA	0.15	1
Rectum Distention	173	2.31	63.01	34.68	279	5.73	68.1	26.17	3.14	0.61
Fecal Matter Color	173	28.32	71.1	0.58	279	46.95	53.05	0	6.94	0.07
Fecal Matter Consistency	173	54.34	41.62	4.04	279	55.91	43.73	0.36	0.34	1
Ventriculus Size	173	6.36	83.24	10.4	279	9.32	78.85	11.83	0.11	1
Pyloric Scarring	173	65.32	34.68	NA	279	95.34	4.66	NA	68.87§	<0.001*
Malpighian Tubule Color	173	98.27	1.73	0	279	98.92	1.08	0	0.03§	0.86
Malpighian Tubule Quantity	173	6.36	93.64	NA	279	21.86	78.14	NA	15.26	<0.001*
Fat body reduction	173	19.65	80.35	NA	279	26.88	73.12	NA	2.67§	0.1
White Nodules	173	87.28	12.72	NA	279	74.91	25.09	NA	7.95	0.04*

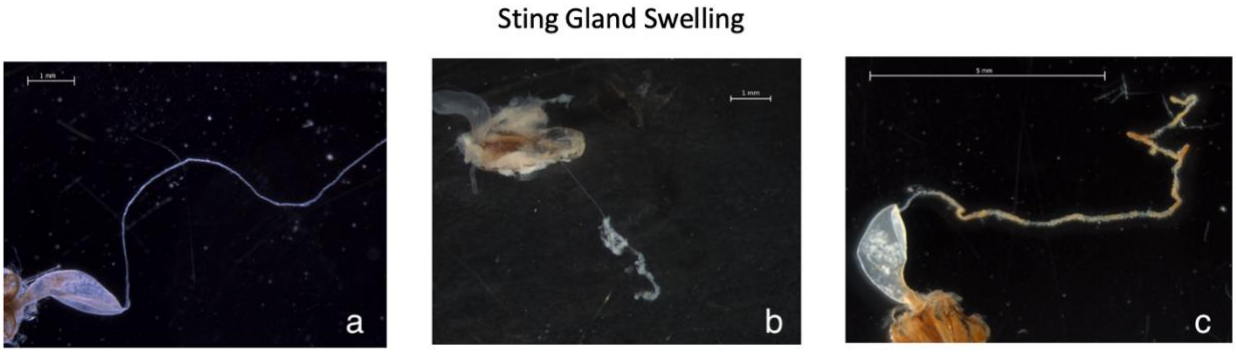

Figure 11*: (a)Normal sized sting gland, (b)intermediate swelling, and (c)severe swelling. a-c; Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23.* *https://doi.org/10.1371/journal.pone.0179535*
b; Photographed by So Eun Moon, 2019

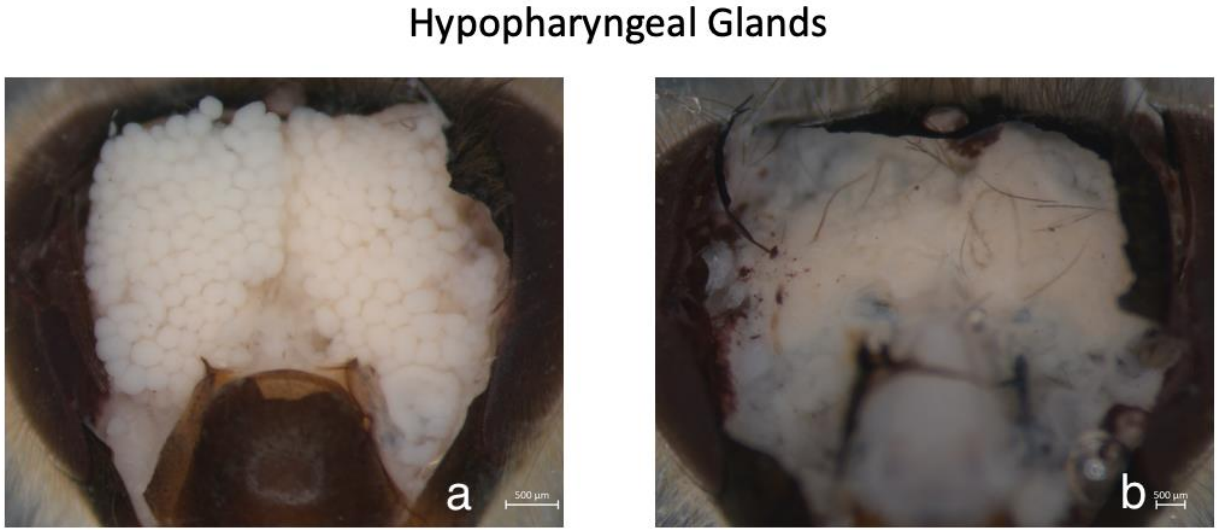

***Figure 12**: (a)Developed Hypopharyngeal glands and (b)atrophied hypopharyngeal gland. (Photographed by So Eun Moon,2018)*

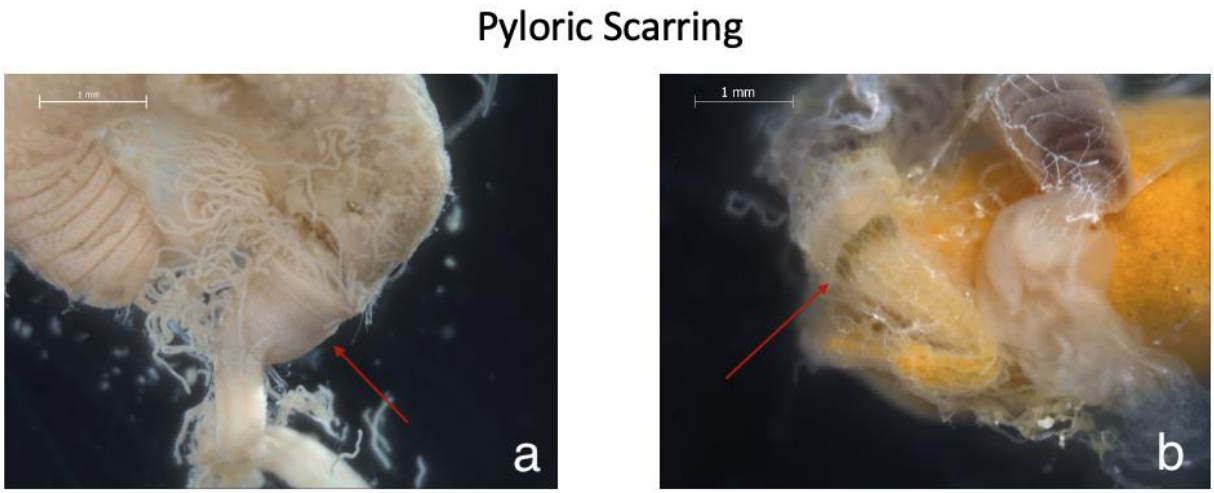

***Figure 13**: (a)Pyloric valve without scarring and (b)pyloric valve with scarring. Adapted from "Colony Collapse Disorder (CCD) and bee age impact honey bee pathophysiology" by vanEngelsdorp et al.,2017, PLoS ONE, 12(7), 1–23. https://doi.org/10.1371/journal.pone.0179535*

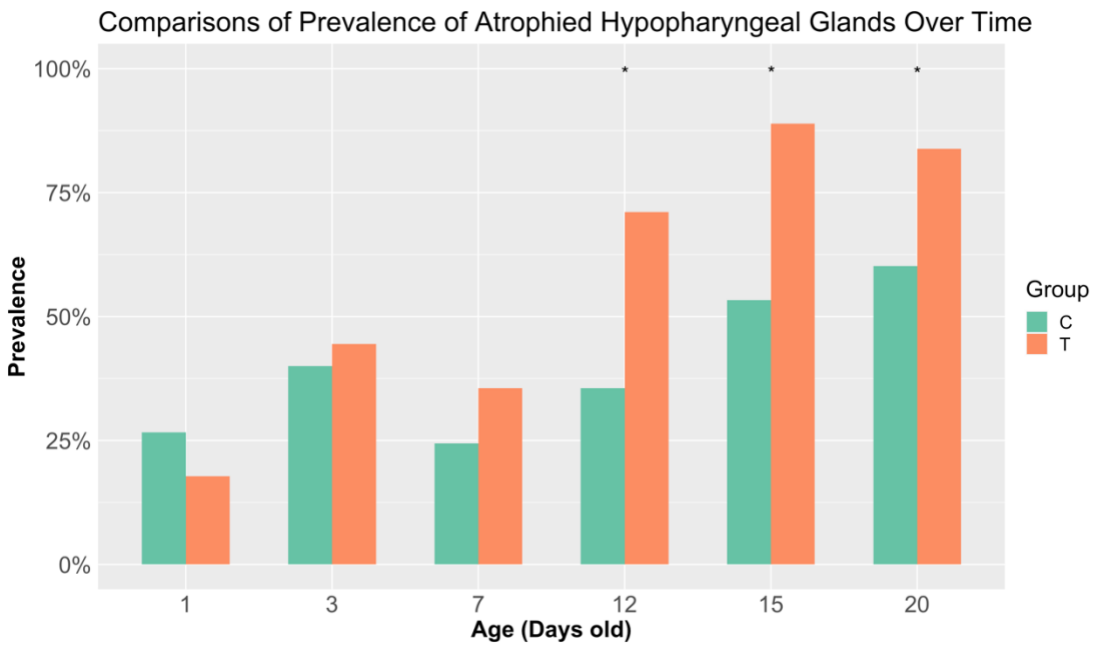

Figure 14: *Comparisons of prevalence of atrophied hypopharyngeal glands over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

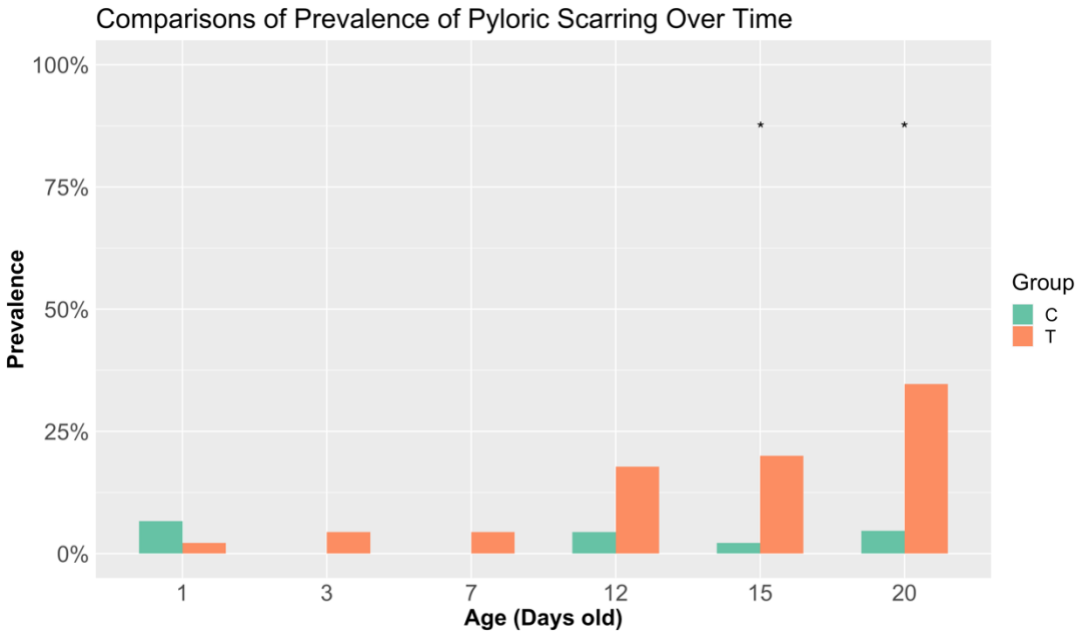

Figure 15: *Comparisons of prevalence of pyloric scarring over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

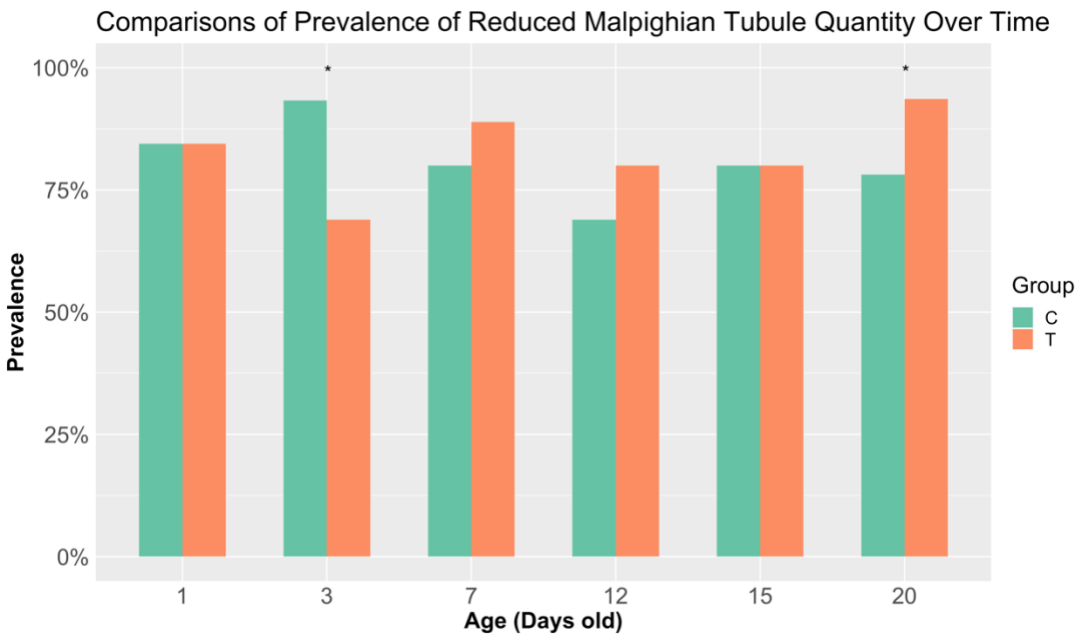

Figure 16: *Comparisons of prevalence of reduced Malpighian tubule quantity over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

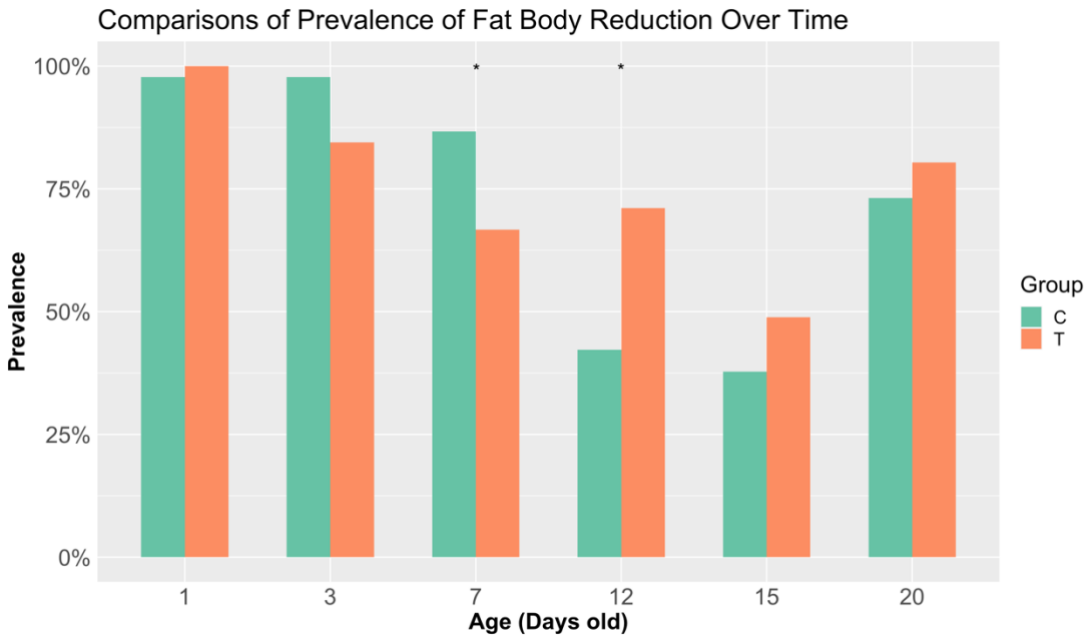

Figure 17: *Comparisons of prevalence of fat body reduction over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

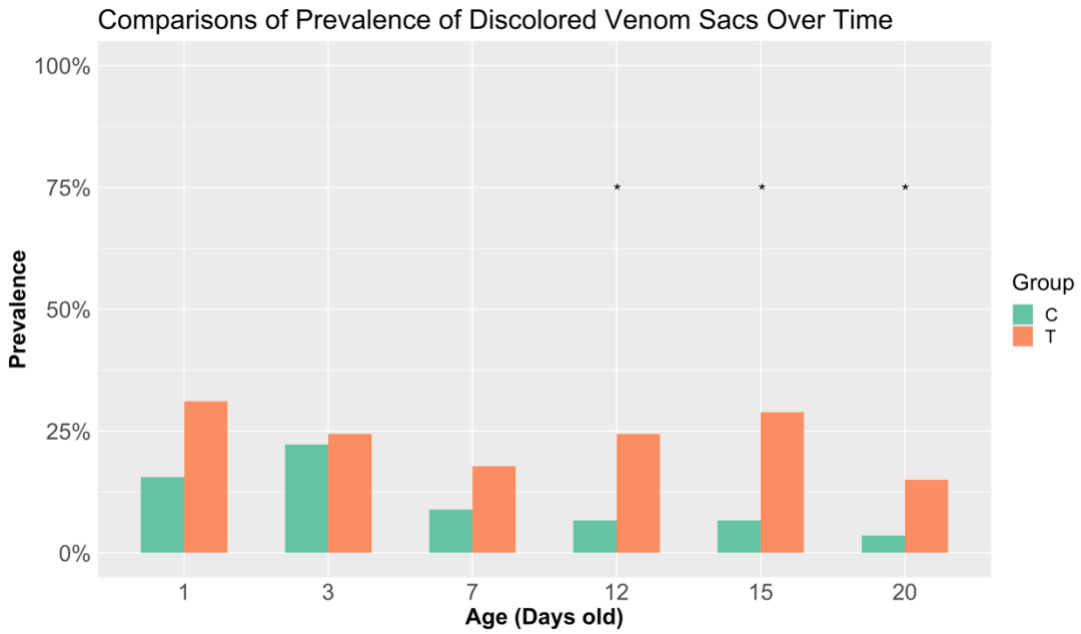

Figure 18: *Comparisons of prevalence of discolored venom sacs over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at α=0.05.*

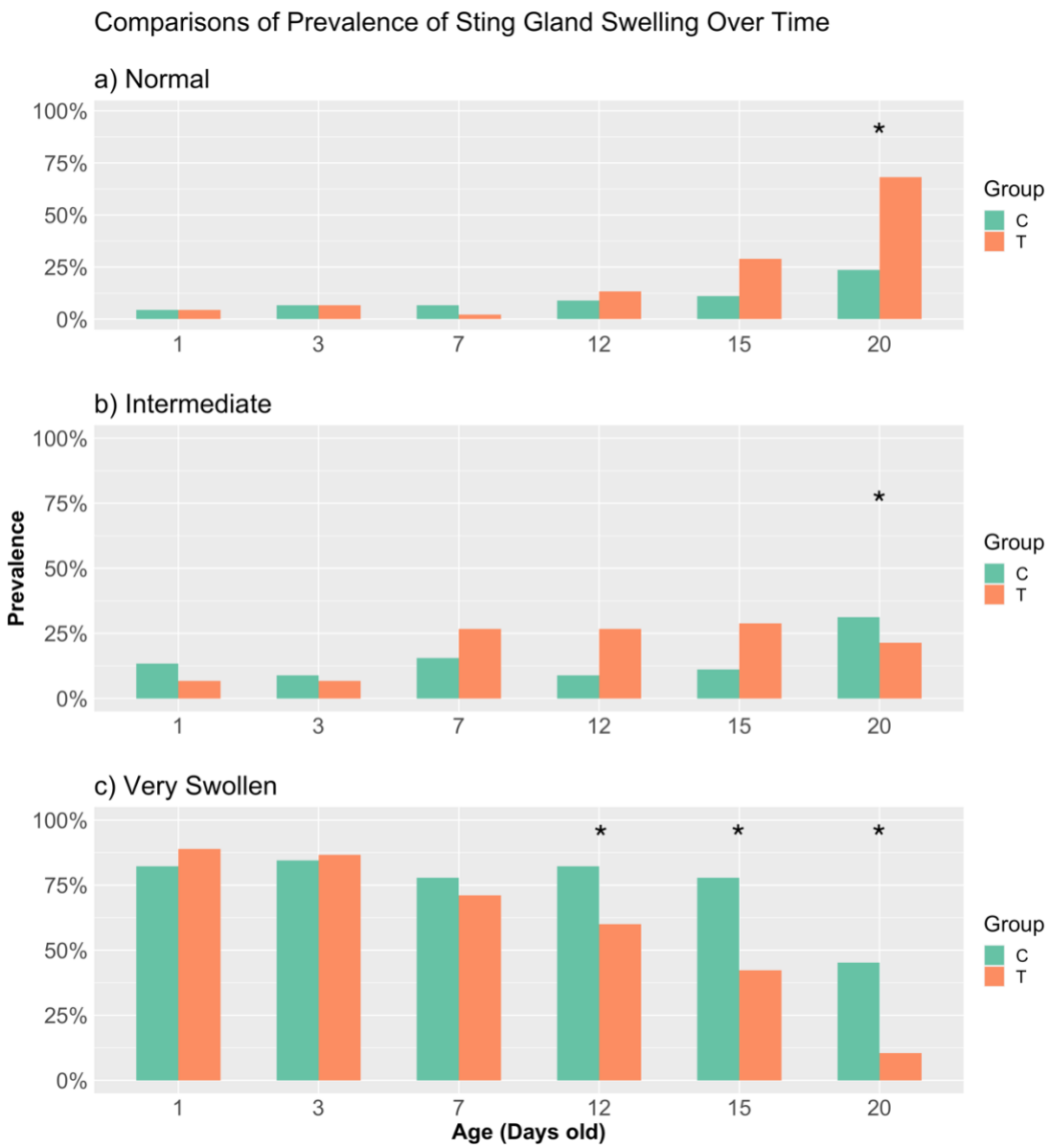

Figure 19: *Comparisons of prevalence of sting gland swelling over time for normal (Table 1; Score 0), intermediate (Table 1; Score 1), and very swollen (Table 1; Score 2) sting glands. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

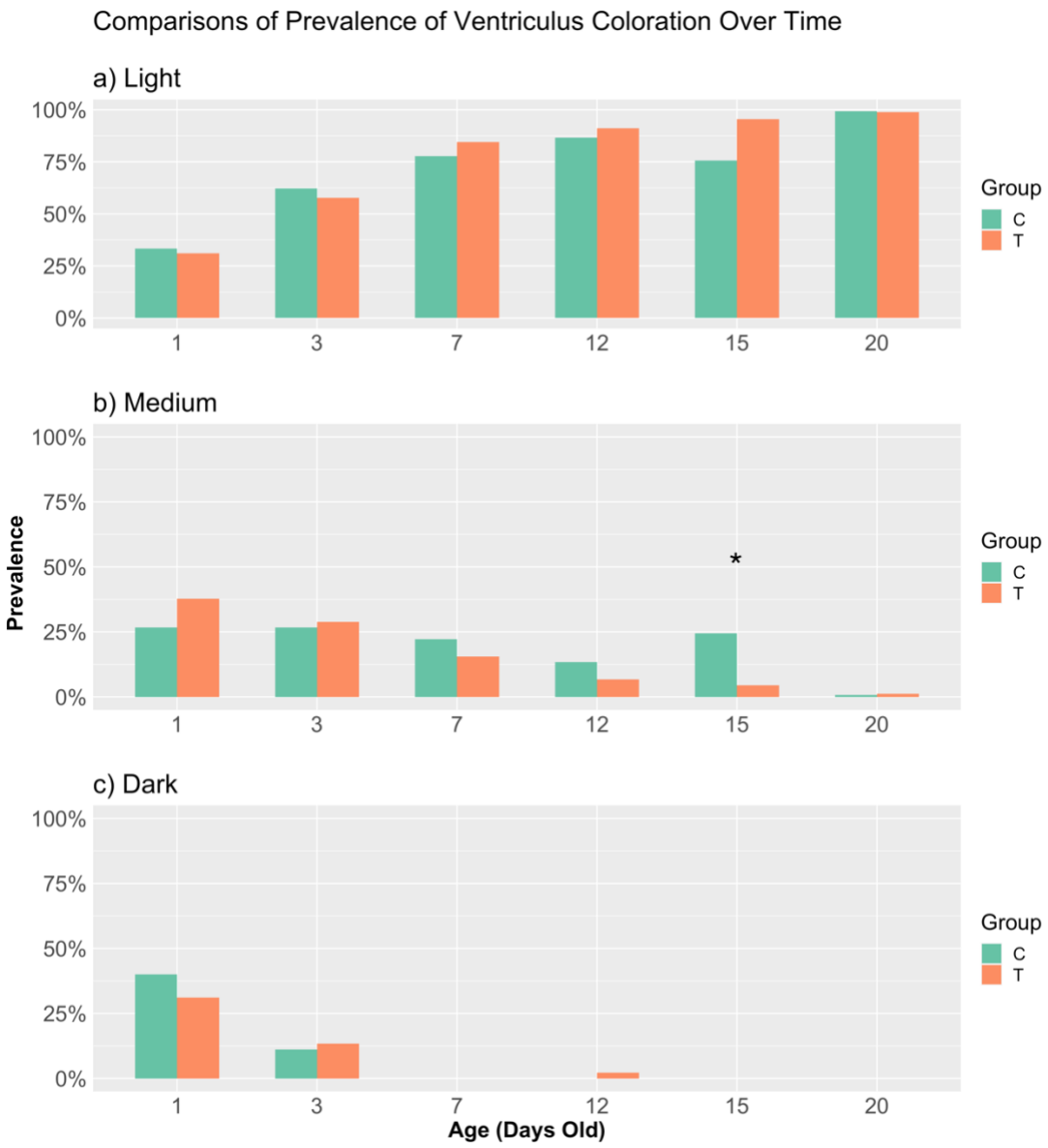

Figure 20: *Comparisons of prevalence of ventriculus coloration over time for light (Table 1; Score 0), medium (Table 1; Score 1), and dark (Table 1; Score 2) colored ventriculi. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

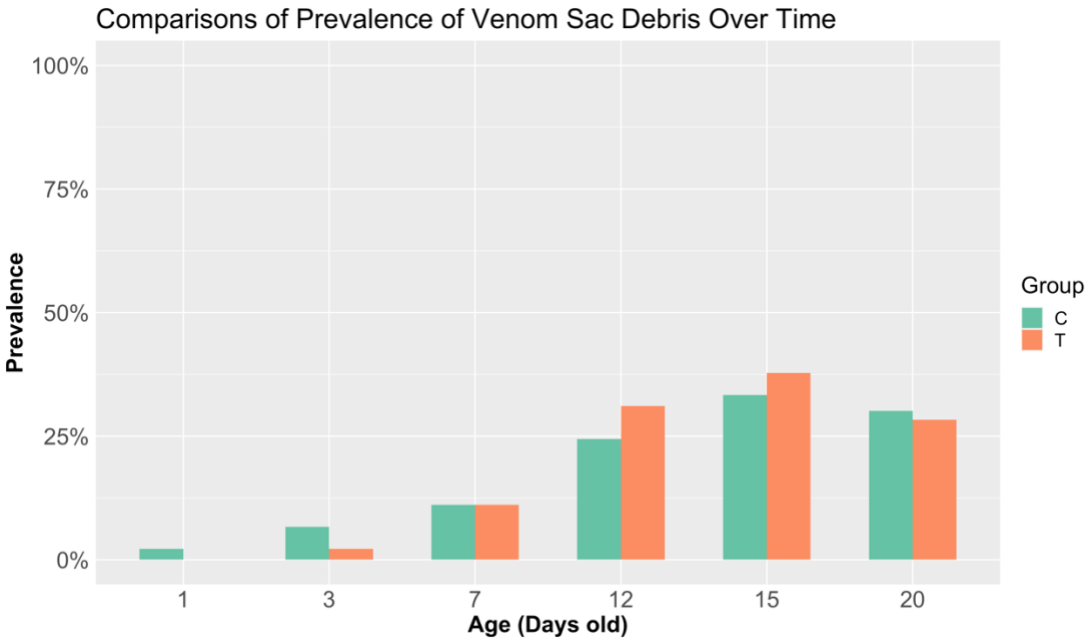

Figure 21: *Comparisons of prevalence of venom sac debris over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. None of the differences between control and treatment groups were statistically significant at* α*=0.05.*

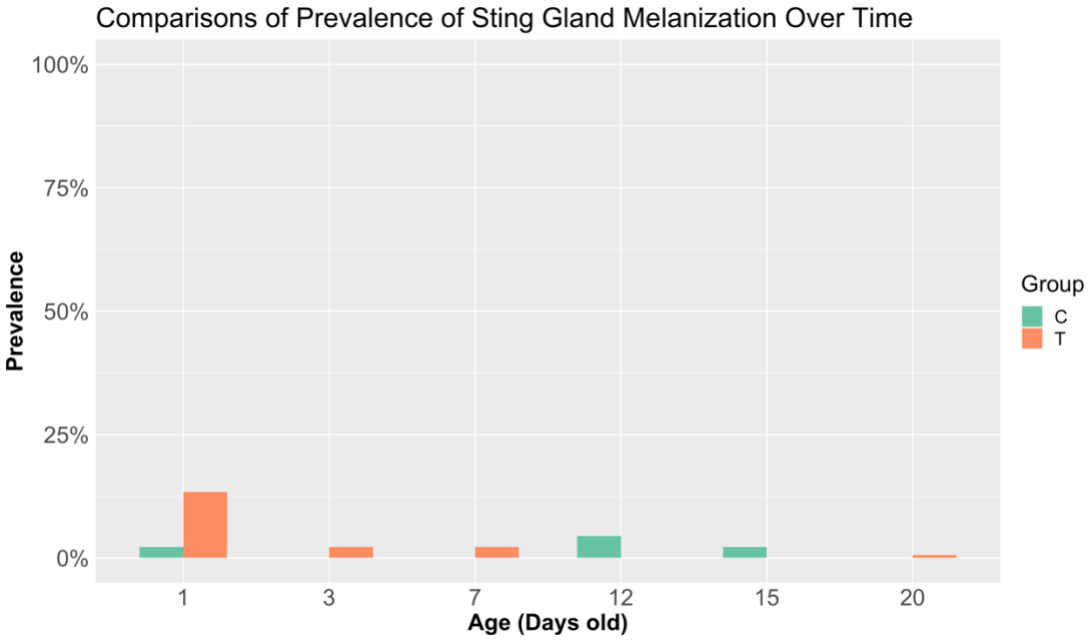

Figure 22: *Comparisons of prevalence of melanized sting glands over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. None of the differences between control and treatment groups were statistically significant at* α*=0.05.*

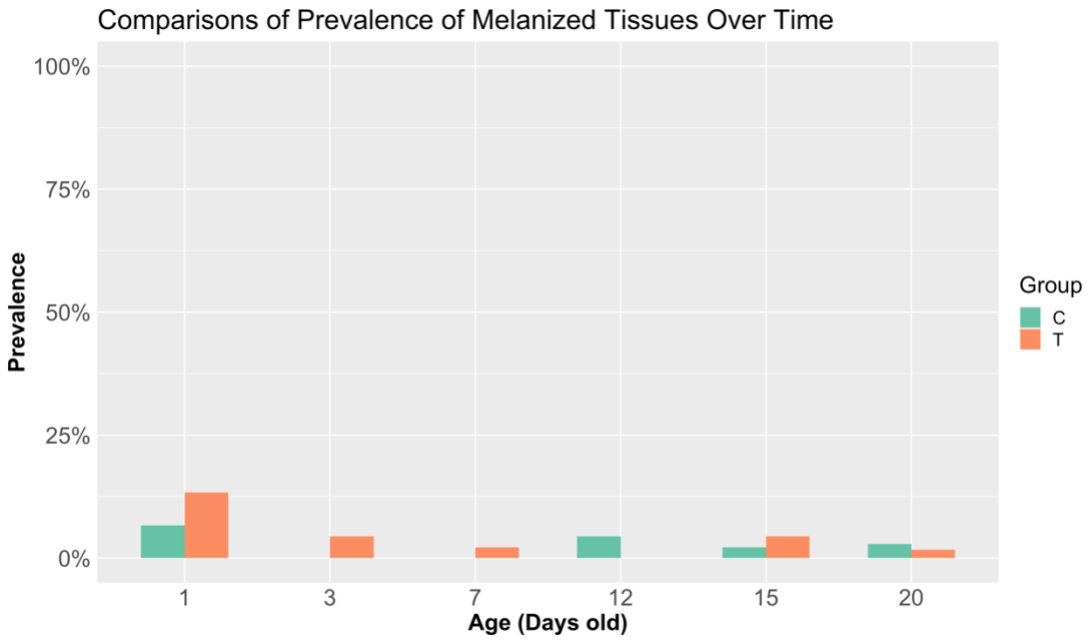

Figure 23: *Comparisons of prevalence of melanized tissues over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. None of the differences between control and treatment groups were statistically significant at* α*=0.05.*

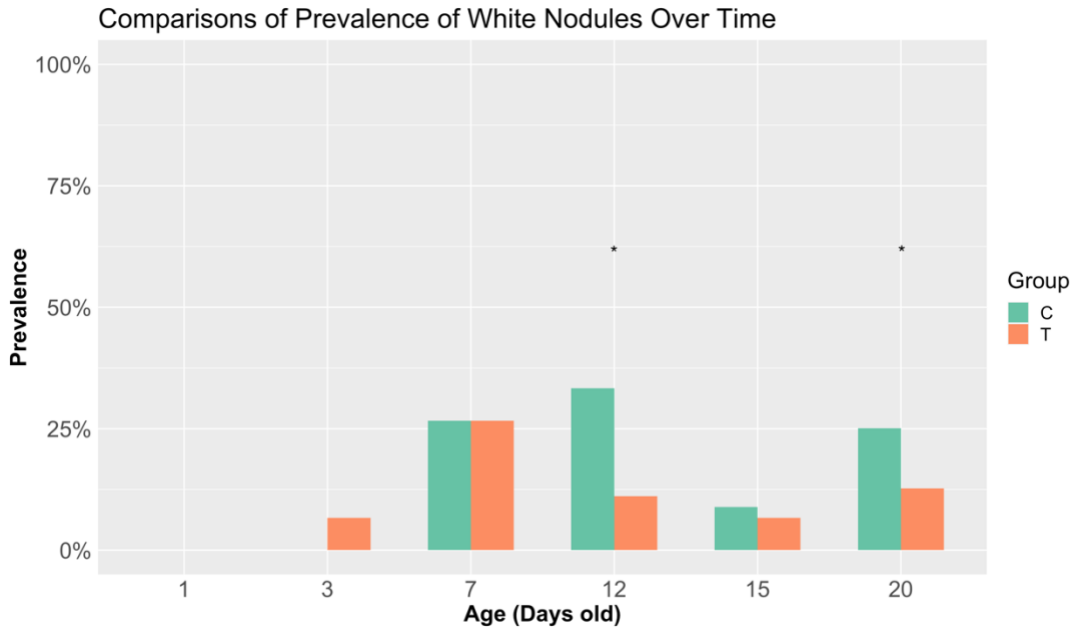

Figure 24: *Comparisons of prevalence of white nodules over time. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

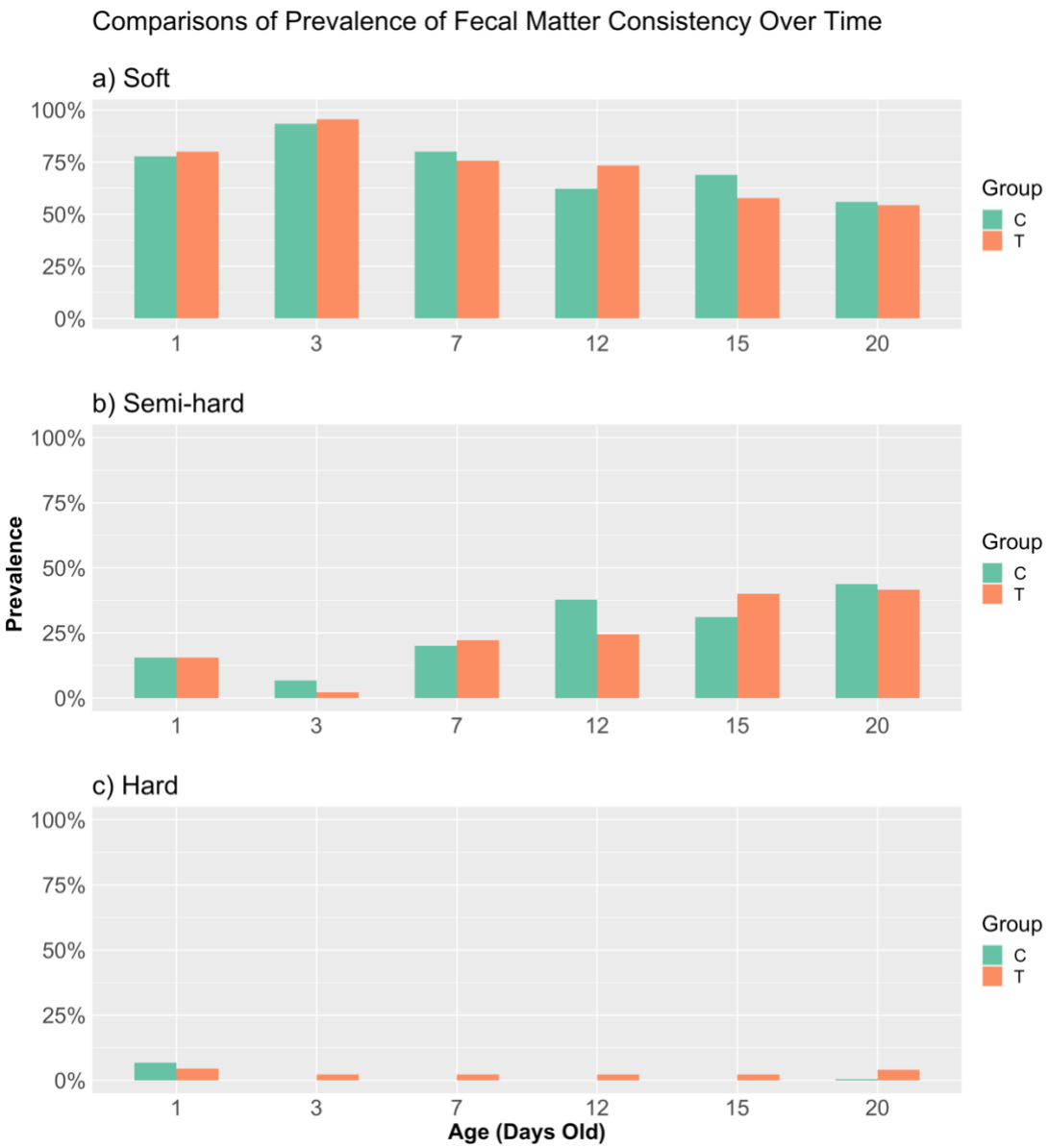

Figure 25: *Comparisons of prevalence of fecal matter consistency scores over time for soft (Table 1; Score 0), semi-hard (Table 1; Score 1), and hard (Table 1; Score 2) fecal matter. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. None of the differences between control and treatment groups were statistically significant at* α*=0.05.*

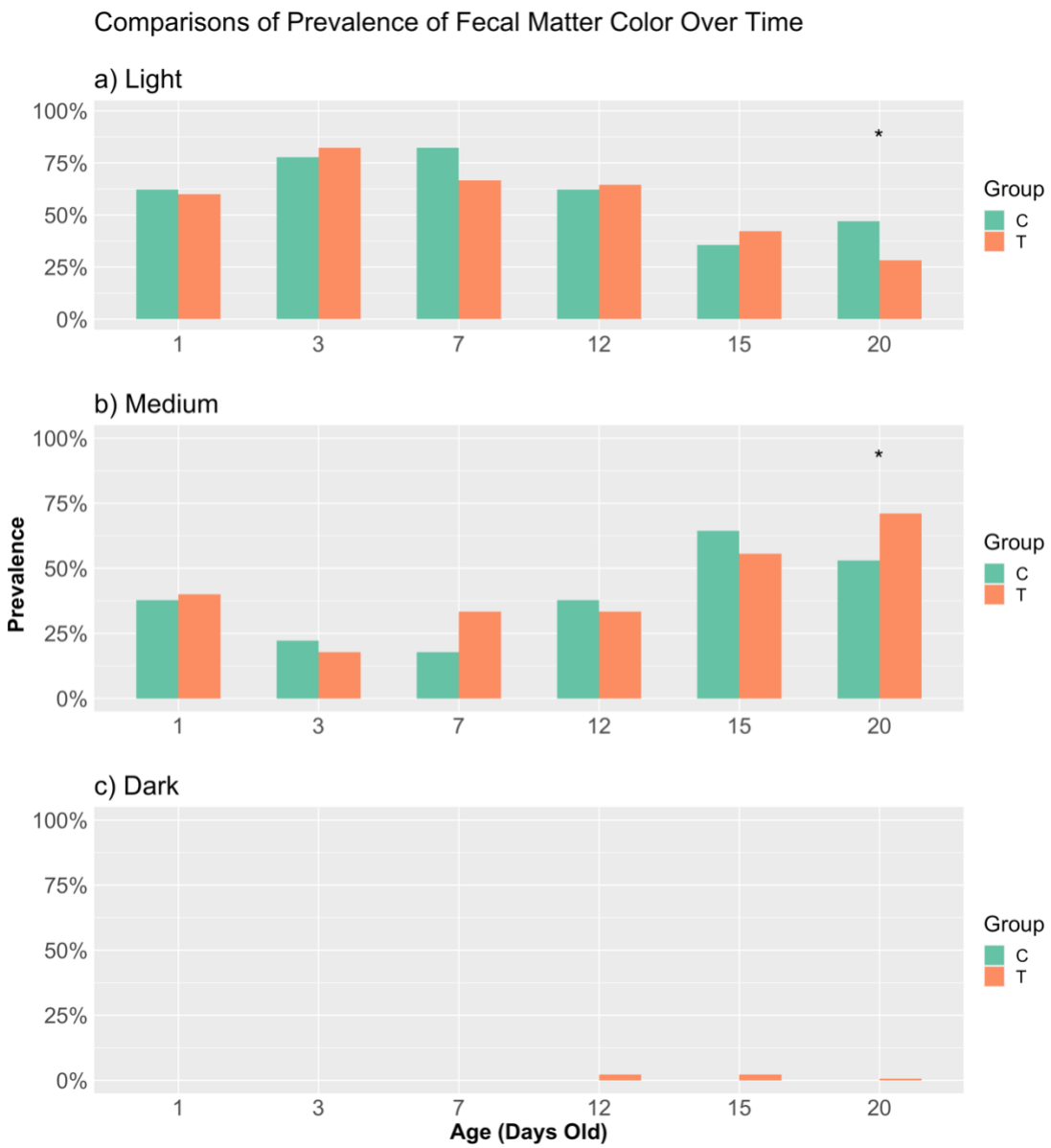

Figure 26: *Comparisons of prevalence of fecal matter coloration scores over time for light (Table 1; Score 0), medium (Table 1; Score 1), and dark (Table 1; Score 2) colored fecal matter. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

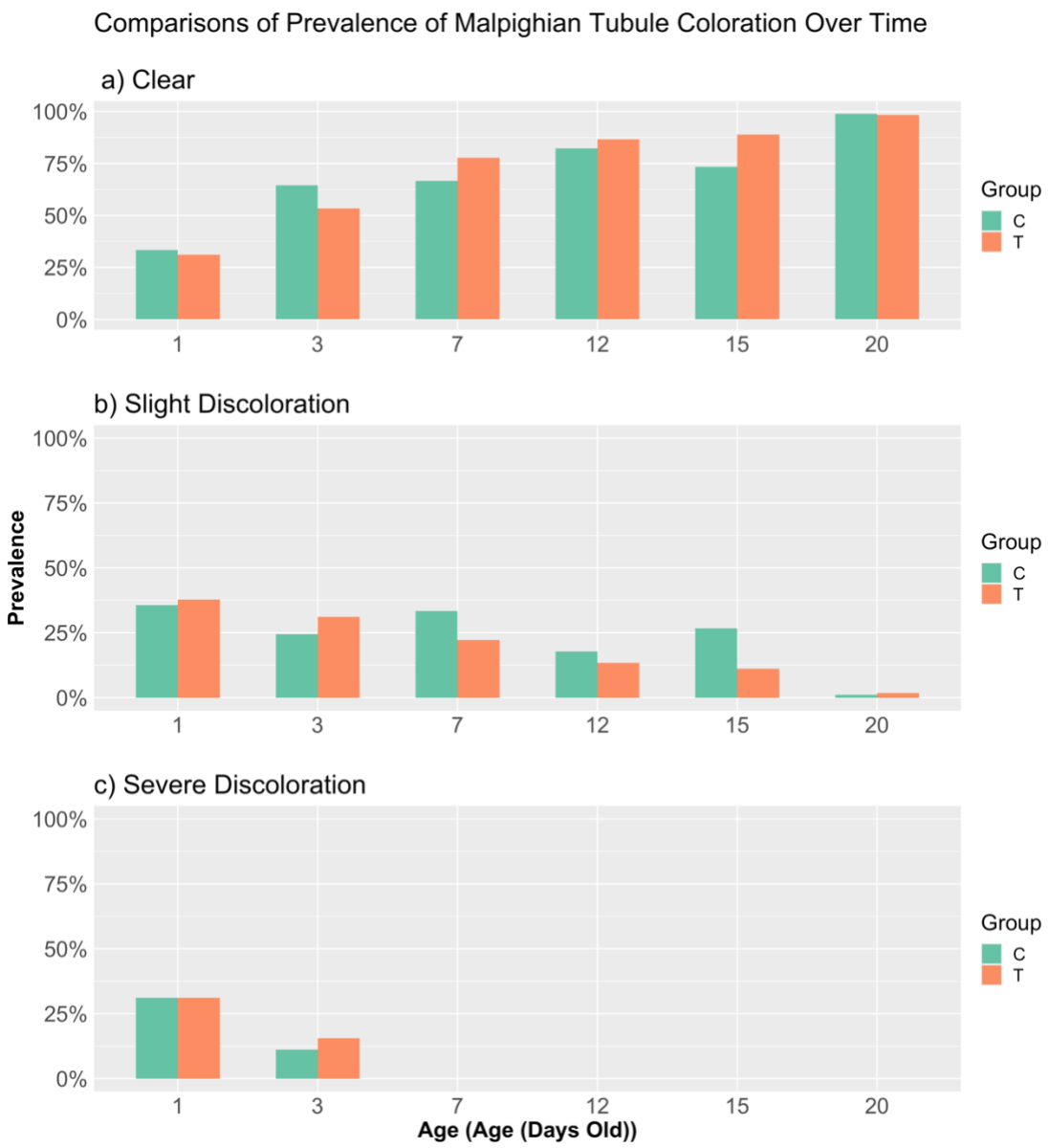

Figure 27: *Comparisons of prevalence of Malpighian tubule coloration scores over time for clear (Table 1; Score 0), slightly discolored (Table 1; Score 1), and severely discolored (Table 1; Score 2) Malpighian tubules. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. None of the differences between control and treatment groups were statistically significant at* α*=0.05.*

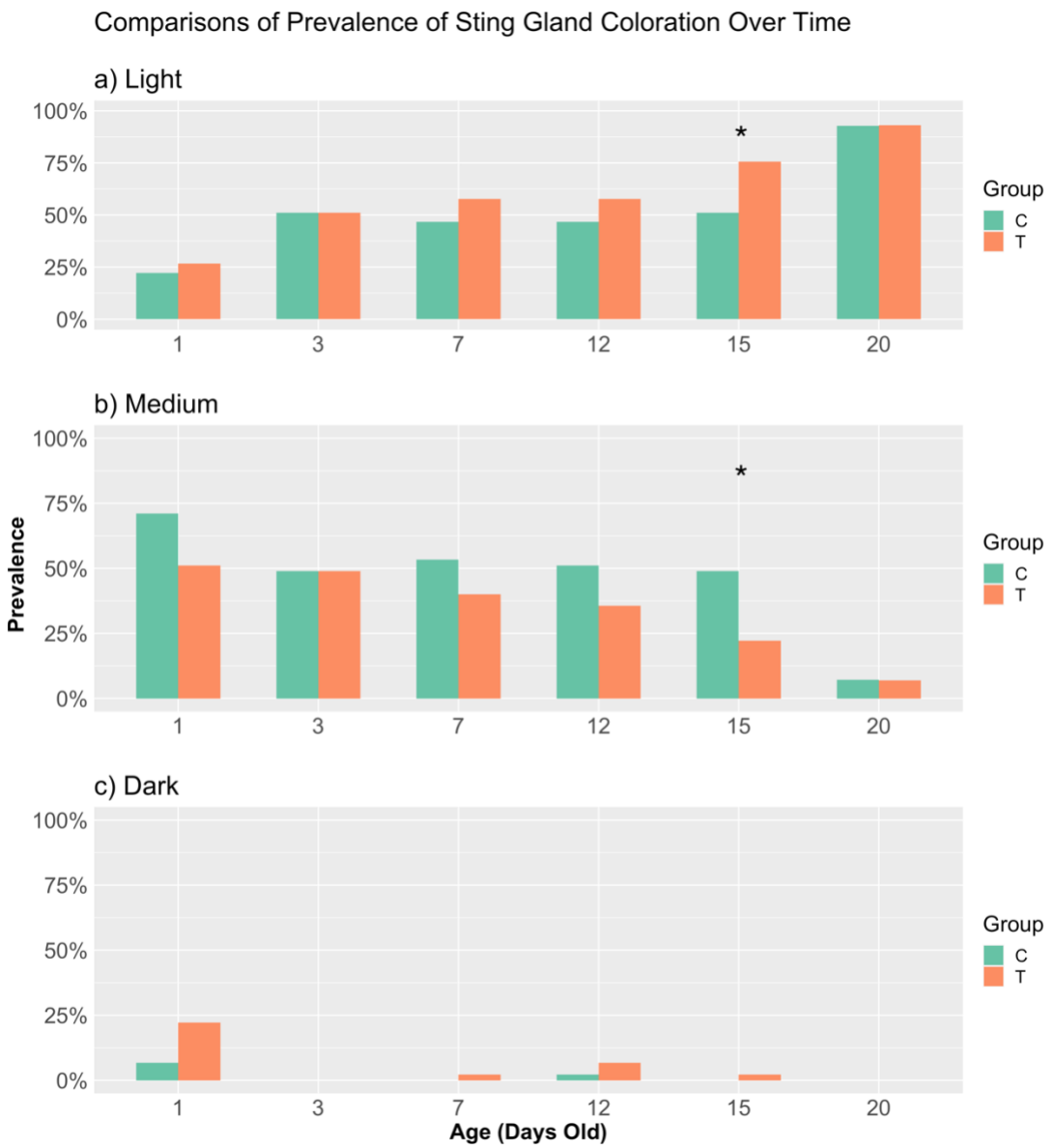

Figure 28: *Comparisons of prevalence of sting gland coloration scores over time for light (Table 1; Score 0), medium (Table 1; Score 1), and dark (Table 1; Score 2) colored sting glands. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. "*" indicates a statistically significant difference at* α*=0.05.*

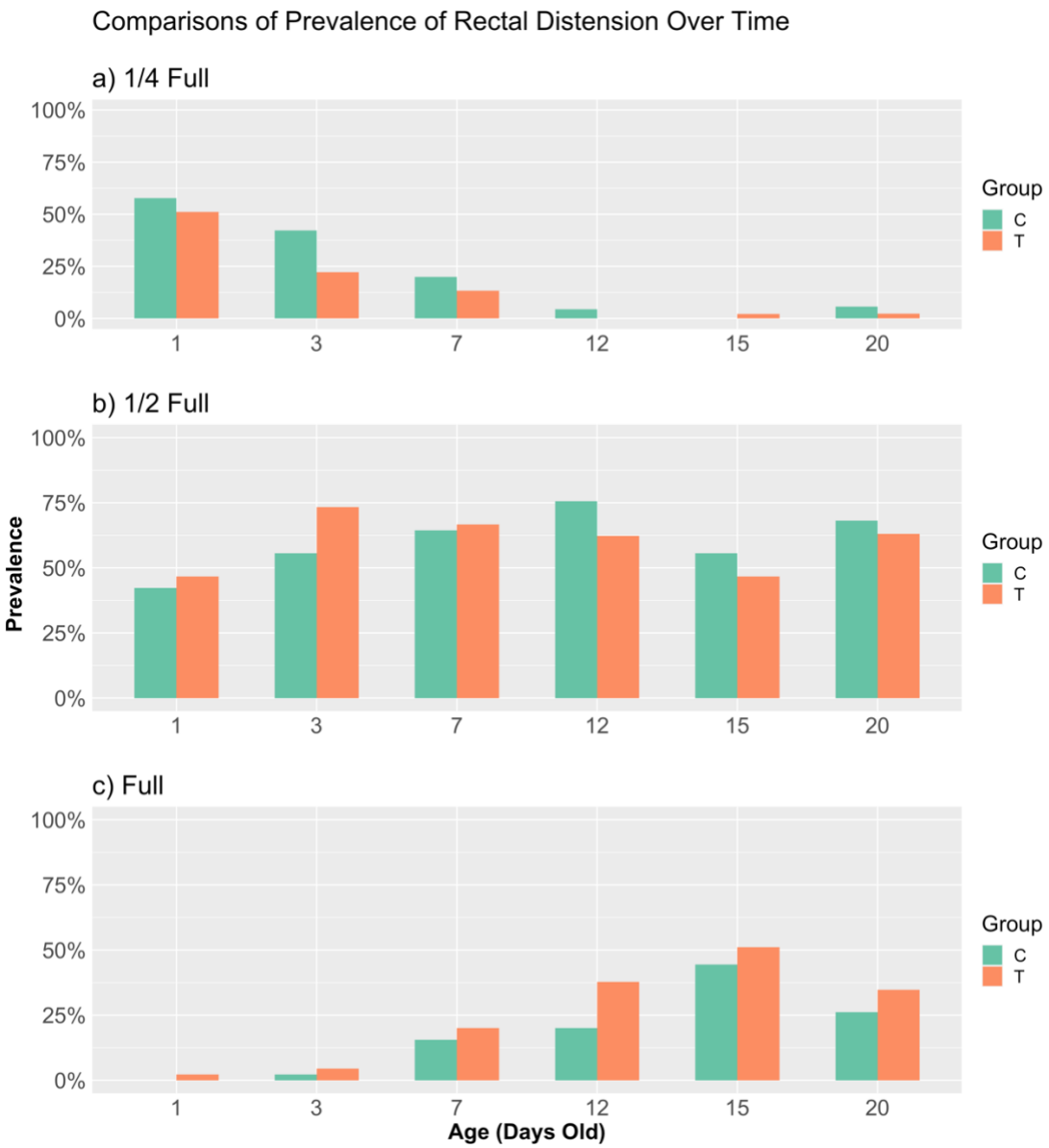

Figure 29: *Comparisons of prevalence of rectum distension scores over time for ¼ full (Table 1; Score 0), ½ full (Table 1; Score 1), and full (Table 1; Score 2) rectums. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. None of the differences between control and treatment groups were statistically significant at* α*=0.05.*

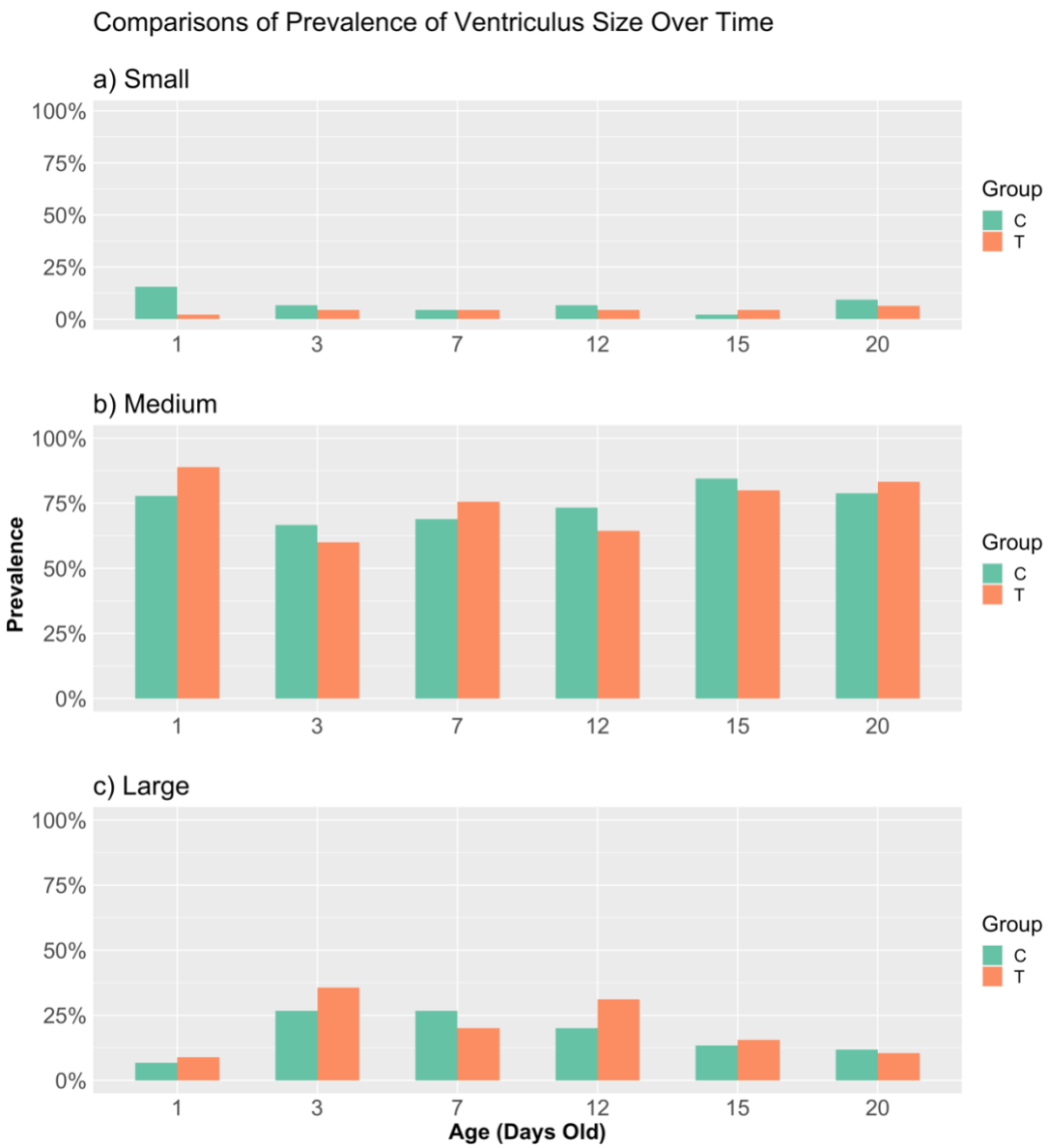

Figure 30: *Comparisons of prevalence of ventriculus size scores over time for small (Table 1; Score 0), medium (Table 1; Score 1), and large (Table 1; Score 2) sized ventriculi. C=Control, T=Treatment. For days 1,3,7,12 control (N=45) and treatment(N=45) groups are equal in sample size. For day 20, control N=279, treatment N=173. None of the differences between control and treatment groups were statistically significant at* α*=0.05.*

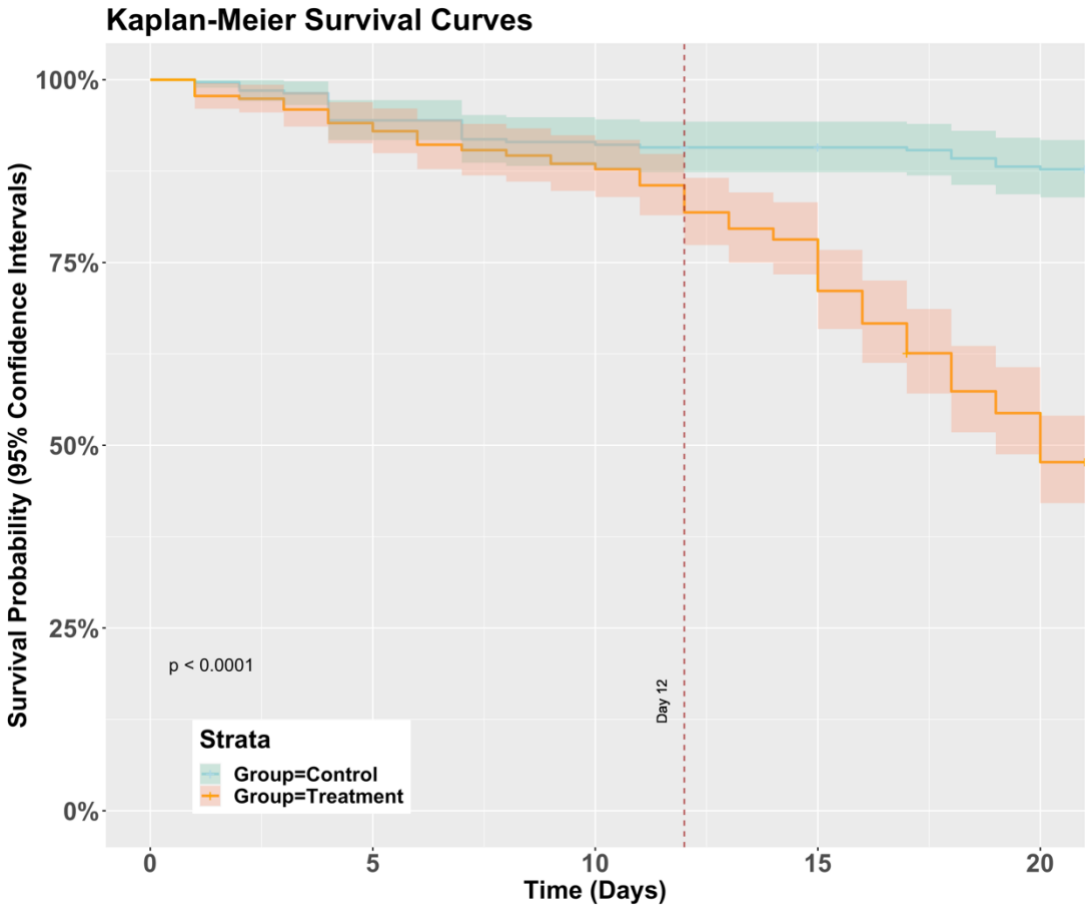

Figure 31: *Kaplan-Meier curves for the control and treatment groups. Nosema infection significantly reduces the survival probability of honey bees (p<0.0001). Vertical line at the day 12, marks the point where the survival probability of two groups becomes significantly different.*

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