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# TRYPANOSOME INFECTION DISTURBS THE GUT MICROBIOTA OF BUMBLE BEE HOSTS

BRYAN SIERRA-RIVERA

43 pages

Host-microbe interactions are ubiquitous with both positive and negative outcomes for host health and fitness. While pathogens abound, there has been an increased focus beneficial host-microbial community associations, such as native gut microbiota that can aid in disease resistance, nutrient sequestration, and detoxification. However, while beneficial microbes can protect against infection, there is a limited appreciation for how pathogen infection may disrupt healthy gut microbiota structure and function. *Crithidia bombi* is a common trypanosomatid gut parasite of bumble bees with fitness-relevant effects on individuals and colonies. The native bumble bee gut microbiota has been shown to increase protection against *Crithidia*, and an association has been found between infection occurrence and host gut microbiota structure in nature. However, we hypothesize that the association between gut microbiota structure and infection could also emerge if infection disrupts the healthy gut community, especially early during the gut community establishment. Using experimental inoculations, we investigate if *Crithidia* infection alters the bumble bee gut microbiota community. *Bombus impatiens* workers were exposed early in microbiota colonization or post establishment to one of three *C. bombi* strains or left naïve. Subsequently, guts were dissected to quantify total bacterial load, numbers of key bumble bee gut bacterial symbionts, and *C. bombi.* We find that infection by *Crithidia* leads to a reduction in total gut bacteria, and when it occurs early during microbiota colonization, *Lactobacillus spp.* and *Gilliamella spp.* numbers are reduced and increased, respectively. This provides evidence for an

infection-mediated disruption of the gut microbiota balance, which could explain microbiota structure and infection associations in the field. Furthermore, healthy microbiota perturbation is another way that pathogens may detrimentally affect hosts, and may underlie some previously described infection effects. This adds to our understanding of the multi-dimensional and directionality of effects between host organisms and their parasitic and beneficial microbes.

KEYWORDS: bumble bee; microbiota; *Crithidia bombi*; multipartite interactions; infection; host-microbe interactions

# TRYPANOSOME INFECTION DISTURBS THE GUT MICROBIOTA OF BUMBLE BEE HOSTS

BRYAN SIERRA-RIVERA

A Thesis Submitted in Partial
Fulfillment of the Requirements
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# TRYPANOSOME INFECTION DISTURBS THE GUT MICROBIOTA OF BUMBLE BEE HOSTS

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B.S.R

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# CHAPTER I: INTRODUCTION

Bipartite interactions have laid the foundation for our understanding of the ecology and evolution of species interactions, but in natural communities we know that interactions are rarely restricted to two partners. More complex multipartite interactions have the potential to alter pairwise species interactions and influence ecological and evolutionary dynamics through direct and indirect effects (Tsui et al. 2015; Tamanash et al. 2022; Guégen et al. 2018; Walsh, 2013; Strauss, 1991). A classic example of such a modification of pairwise interactions is in rocky intertidal communities where there is competition for space between mussels (*Mytilus californianus*) and barnacles (*Balanus glandula*) (Paine, 1966). Mussels are dominant competitors and have a direct negative effect on barnacles, the inferior competitor. However, this dynamic changes in the presence of a predatory sea star (*Pisaster spp.*), due to its selective feeding on mussels. This direct negative effect on the mussel population by the sea star provides competitive release for the barnacles, and thus has an indirect beneficial effect on the barnacle population. Ecological and evolutionary effects on species within these more complex multipartite interactions have frequently focused on larger macro-scale organisms, while tripartite interactions between host organisms, beneficial microbes associated with them, and infectious agents have received less attention. However, such interactions have the potential to significantly affect host health and the dynamics of infectious disease.

Communities of host-associated microorganisms are termed the microbiota. Members of the native microbiota play important roles in determining the health and fitness of their hosts. The appreciation and understanding of the associations between hosts and their beneficial microbes have increased in recent decades, with a major focus being microbes within the gastrointestinal

tract of animals known as the gut microbiota. The importance of the gut microbiota is seen widely across taxonomical groups, including termites (Brune & Dietrich, 2015), social bees (Kwong & Moran, 2015), the Hawaiian bobtail squid (Visick et al. 2000), zebrafish (Flores et al. 2020), mice (Kostic et al. 2013), and humans (Sekirov *et al.,* 2010; Dave et al. 2012). Bacteria within these gut microbe communities can positively influence host health by aiding in disease resistance and tolerance (Hooper et al. 2012; Duffy et al. 2007; Schmid-Hempel, 2021), nutrient sequestration (Engel & Moran, 2013) and detoxification of various chemicals (Kešnerová et al. 2017). Understanding colonization by and maintenance of beneficial microbes is of particular importance since dysbiosis of a host's microbiota caused by antibiotics (Raymann et al. 2017), diet (Muegge et al. 2012), and infection (Sekirov et al. 2010) can reduce function with knock-on effects for host health and fitness.

While studies on beneficial microbe associations and interactions with their hosts have only gained traction more recently, considerable research effort has been dedicated to parasitic microbes including their evolutionary and ecological interactions with hosts (Woolhouse et al. 2002; Harvell, 2004). These parasitic microbes take advantage of the host environment for their own gain, to the detriment of the organism they infect. While by definition parasites negatively impact host fitness, infection outcomes for hosts vary due to different mechanisms of pathogenesis. For example, infection can directly impair host behavioral functions, such as mite infected barn swallows (*Hirundo rustica*) that have decreased aspects of flights compared to uninfected individuals (Barbosa et al. 2002), cause direct tissue destruction, as with *Entamoeba histolytica* in humans (Ghosh et al. 2019), or impart indirect harm by behavioral manipulation of hosts, as shown in *Toxoplasma gondii* infected mice that exhibit decreased anti-predator avoidance to cats (Berdoy

et al. 2000). These and other negative effects on hosts can significantly affect host populations and their evolution, making understanding factors contributing to variation in infection and infection outcomes of high relevance.

Microbial colonization by both beneficial microbes and pathogens may be affected by host associated factors, such as diet or host physiology. In the gut of mammalian hosts, microbiota feed on glycans which are both ingested by the host and secreted by host cells (Koropatkin et al. 2012). As the mammalian hosts progresses from milk feeding young, towards adulthood, the glycan composition in the gut shifts, resulting in gene expression changes by both the microbes residing in the gut and host cells (McKeen et al. 2019). When looking at diet and infection, in a bumble bee, *Bombus terrestris*, higher gut trypanosome infections result when the adult host is allowed to feed on pollen (Logan et al. 2005). Interactions of microbes with host immunity, including its activation or evasion, can also mediate apparent competition for colonization outcomes of microbes (Hoebe et al. 2004; Schmid-Hempel, 2021; Armitage et al. 2022). The innate immune system utilizes rapid, often non-specific responses, and many components, including recognition receptors, phagocytosis, and antimicrobial compounds, have been shown to be upregulated in the presence of pathogens or normal gut-associated microbes (Rosales et al. 2017; Rus et al. 2005; Mukherjee et al. 2008; Li et al. 2012; Kwong et al. 2017). The adaptive immune system also responds to both pathogens and the normal gut microbiota (Bonilla & Oettgen, 2010; Feng & Elson, 2011; Zhang et al. 2015). In turn, colonizing microbes can also modulate host immunity, altering host responses, as seen in a plant-fungal pathogen system where fungal oomycetes can reduce expression of immune stimulating genes (Na & Gijzen, 2016; Houterman et al. 2008) or even hijacking host responses (Hornef et al. 2002). The interactions and responses noted above

between hosts and their associated microbes, pathogenic or beneficial, will drive eco-evolutionary dynamics for the organisms involved (Macke et al. 2017; Salathé et al. 2008; Greischar & Koskella, 2007; Suzuki & Ley, 2020; Obeng et al. 2021). There are, however, several other mechanisms through which pathogens and normal microbiota may interact.

While both theory and empirical work has accumulated on direct interactions between hosts and their pathogenic or beneficial microbes, there can be interactions between colonization and maintenance of beneficial microbes and pathogen infection, including a variety of non-competitive effects and competitive effects (Armitage et al. 2022). Competitive mechanisms include exploitative competition, interference competition, and apparent competition. Exploitative competition is direct competition due to a shared demand for limited resources, such as nutrients or space. Bacteria in the gut tend to form thick biofilms that can prevent pathogens from interacting with the host epithelium, providing protection via colonization resistance (Stecher & Hardt, 2011). This can also come about by restricting access to iron acquired by both pathogenic and beneficial microbes (Chen & Ayres, 2020; Sassone-Corsi & Raffatellu, 2015). Interference competition involves direct antagonistic interactions between pathogens and beneficial microbes that reduce the competitor's fitness. This can include chemical warfare, and pathogens have evolved secretion systems, virulence factors, and extracellular antimicrobial molecules such as bacteriocins to combat beneficial microbes (Riley & Wertz, 2002; Heine et al. 2018). Apparent competition between beneficial microbes and pathogens is indirect competition mediated by the host immune or other response. In mice, *Salmonella* and *Citrobacter* infection can induce the host inflammatory response of the intestines and disrupts the microbial community (Barman et al. 2008; Lupp et al. 2007). Infection of ticks by a bacterial pathogen induces expression of an anti-freeze glycoprotein

altering gut microbiota biofilm formation and thus the gut microbial community (Abraham et al. 2017). Although our knowledge is growing, as shown in these examples, while a native microbiota can protect against parasite infection, there remains a limited appreciation for how pathogen infection can disrupt a healthy gut microbiota structure and function outside of mainly mammalian systems. Understanding the full extent of these interactions between pathogenic and beneficial microbiotas is necessary to understand infection dynamics and host fitness in systems where host-associated microbes have important consequences for host health.

Corbiculate bees, a clade that includes the social honey bees (*Apis* spp.) and bumble bees (*Bombus* spp.), have become important systems for understanding host-microbiota interactions in general and in the context of the health of these important pollinators (Engel et al. 2016; Kwong & Moran, 2016; Zheng et al. 2018; Wang et al. 2019; Romero et al. 2019). In these systems it appears that interactions between beneficial microbes and pathogens could have important consequences. In nature, an association has been demonstrated between bumble bee gut microbiota structure and infection with a gut trypanosome pathogen of bumble bees, *Crithidia bombi* (Cariveau et al. 2014; Felden et al. 2021; but see Li et al. 2015). Occurrence of pathogen infection has been found to be negatively associated with the presence of a specific bumble bee gut microbe (Cariveau et al. 2014) and a more recent study found wild caught *B. terrestris* infected with *Crithidia* to have more diverse and distinct gut microbial structure relative to uninfected bees (Felden et al. 2021). This pathogen and host-microbiota association could be the result of two non-mutually exclusive processes. Either *Crithidia* infection alters the native microbiota or differences in the native microbiota and its structure determine *Crithidia* infection outcomes. Studies have elucidated support for the second reasoning, with the presence of a native healthy gut microbiota

in bumble bees providing resistance to *Crithidia* infection in bumble bees (Koch & Schmid–Hempel, 2011) and microbiota transplants between host colonies indicating the microbiota can be important in determinant of infection differences (Koch & Schmid-Hempel, 2012). Experimental manipulations of the gut microbiota have revealed that more diverse microbiotas and the presence of *Apibacter*, *Lactobacillus* Firm-5, and *Gilliamella* in the gut community lead to lower *C. bombi* infections (Mockler et al. 2018). However, we still do not know if infection by *Crithidia bombi* alters colonization or maintenance of gut microbiota membership. Such an effect could disrupt host health by the indirect effect of microbiota dysbiosis associated with infection and a subsequently altered gut microbiota function.

Host-specificity of gut bacteria associations in social bees are suggested to be ancient and exhibit similarities to associations of humans and their microbes (Kwong & Moran, 2016). Social bees also serve as a tractable system for investigating the evolution and ecology of host–microbe interactions since they are host to a relatively simple and consistent microbial community of about 13 to 15 core members which are consistent between species (Kwong & Moran, 2016; Martinson et al. 2011). Within the social bee gut microbiota, *Snodgrassella alvi,* a beta-proteobacterium, is acquired through vertical transmission from mother queens (Koch et al. 2013; Martinson et al. 2011). *Snodgrassella alvi* is one of the first microbial species to settle in the adult hindgut, directly interacting with the host's gut epithelium (Katsnelson, 2015) and forming a foundation for the further development of the microbiota (Lim et al. 2015). Further gut colonizers *Gilliamella spp.* and *Schmidhempelia bombi* aid in metabolism of complex carbohydrates (Engel et al. 2012; Zheng et al. 2016; Lee et al. 2015) while *Lactobacillus spp*. aid with fermentation of simple carbs (Zhang et al. 2022). For the microbiota as a whole, and individual members within it, the beneficial effects

on host health extend beyond the protection against pathogen infection outlined above and include a positive effect on weight gain through metabolism of pollen, the regulation of gut physiology (Zheng et al. 2017), the metabolism of toxic sugars (Zheng et al. 2016), and a contribution to immunological development (Kwong et al. 2017). On the other hand, bumble bee hosts face many pathogens that have detrimental effects on host health (Cameron & Sadd, 2020). *Crithidia bombi* (Lipa & Triggiani, 1988) is an obligate gut infecting trypanosome and is transmitted via feces both within bumble bee colonies but also to other individuals within the bee community through shared flower use (Durrer & Schmid-Hempel, 1994; Koch et al. 2017). Infection by *C. bombi* has a variety of fitness relevant consequences for bumble bee hosts such as increased mortality under stress, reduced worker longevity, impaired foraging success, reduced queen hibernation survival and colony founding success, and negative impacts on overall fitness (Cameron & Sadd, 2020; Gegear et al. 2005; Gegear et al. 2006; Fauser et al. 2017; Sadd & Barribeau, 2013). Such negative impacts could arise from or be reduced by changes of the beneficial gut microbiota of bumble bees upon infection with *Crithidia.*

Using the experimental infection system of the *B. impatiens* and *C. bombi,* we investigate how gut infection by the trypanosome pathogen at two points of native microbiota establishment influences the abundance of key members of the bumble bee host gut microbial community. I hypothesize that, because of the direct or indirect effects of *C. bombi* infection, core gut symbionts will be perturbed in the bumble bee gut, resulting in changes to the gut microbial community. In addition, I hypothesize that the timing of infection in relation to native gut microbiota establishment will affect the extent of this perturbation. I predict that the microbiota will be more susceptible to infection induced changes early in its colonization. Establishing an understanding

of the directionality of effects in multiple host-microbe interactions, such as that of bumble bees their beneficial microbes and pathogens, is important for understanding associations described between them in nature and how cascading effects based on interactions between microbes may impact host health.

# CHAPTER II: MATERIALS AND METHODS

## Bee husbandry and overall approach

Five commercial bumble bee (*Bombus impatiens*) colonies (Koppert Biological Systems, Howell, MI, USA) were transferred to custom lab hives to acclimate for 7 days. Colonies were held under red-light at 26±2°C, provided with 50% sugar water (1 gram of cane sugar, 1 mL of boiled water and 0.1% cream of tartar) *ad libitum* and provided with given honey bee collected pollen (Swarm Bustin Honey, West Grove, PA) three times a week. Subsequently isolated adult workers were kept under the same conditions but provided with Ethylene Oxide (ETO) gas sterilized organic wildflower honeybee pollen (Matt Messa's Farms, Wisconsin) and the same sugar water. Original queens and a random subset of workers were screened for common gut pathogens and all colonies were deemed pathogen-free. Post colony acclimation, all workers were removed from the colony and a subset were marked with white out on the top of the thorax and returned to the colony. This procedure allowed newly emerged workers to be distinguished. Newly emerged callow workers (<24 hours after emergence) were removed from the colonies and given a feces mixture from their native colony to standardize exposure to gut microbes. At day 0 (early) or day 4 (late) during microbiota establishment they were either left naïve as pathogen-free controls or experimentally exposed to one of three *C. bombi* strains (AK 08.052, CH 08.075, IL 16.075). This gave eight treatment combinations of pathogen exposure and timing (Figure 1).

## Culturing *Crithidia bombi*

Three strains of *C. bombi*, previously isolated from wild bumble bee populations were used for this experiment. Strain CH 08.075 was collected and isolated from Switzerland in 2008, strain IL 16.075 was from Central Illinois, and was isolated in 2016, and strain AK 08.052 was from

Alaska in 2008. These strains were derived from single parasite cells, were confirmed as *C. bombi* by molecular markers, and are maintained in a frozen strain bank at -80°C, following previous methods (Salathé et al. 2012). Fifty percent glycerol stocks were prepared with 300 μL of glycerol and 600 μL of *Crithidia* cell culture. To have viable *C. bombi* cells available for experimental exposures, strain stocks were thawed twice weekly to inoculate fresh FP-FB media and cultured at 27°C and 3% $CO_2$ (Salathé et al. 2012). Strains from thawed 50% glycerol stocks were cultured in 7 mL of full FP-FB medium (5.66 mL of FP stock solution, 0.63 mL of sterile filtered 10x FB stock solution, 0.71 mL of heat inactivated fetal bovine serum, and 7 μL of haemin). *C. bombi* strains were cultured in duplicates.

**Fecal inoculum collection**

Aged (>48 hours after emergence) marked workers from each colony were removed and placed into holding vials and agitated to collect fresh feces. Feces were collected with 10 μL microcapillary tubes and transferred to a sterile 1.5 mL microcentrifuge tubes. Feces from workers from the same colony were pooled until the required volume was acquired. Feces from a minimum of five workers per colony were combined to reduce heterogeneity in the fecal inoculum pool for each colony.

**Fecal transplants and *Crithidia* exposure**

Age controlled (within 24 hours of adult emergence) experimental adults were placed into holding vials and starved for one hour before receiving 15 μL of a 2:1 mixture of sugar water and collected feces from their source colony. Early pathogen exposure (day 0) bees were subsequently starved for an additional hour before being presented with 10 μL of their respective *Crithidia* strain

inoculum or a parasite-free solution for controls. Late pathogen exposure (day 4) bees were given the microbiota inoculum and then placed into their respective individual boxes with pollen and sugar water after a further hour. Four days post microbiota inoculation, these workers were given their designated *Crithidia* strain or left as un-exposed controls, as above. *Crithidia* inoculums were prepared from *in vitro* cultures (3-4 days following their initiation). Culture cell densities were quantified using Globe Scientific Quick-Read Urinalysis System slides and then immediately before experimental pathogen exposure took place diluted in sugar water to give a final concentration of 10,000 cells per 10 μL. After inoculums were provided, all bees were visually monitored to ensure the treatment was completely consumed. Any bees that did not consume the inoculum within 60 minutes were removed from the experiment. After inoculation was confirmed visually, bees were returned to their individual boxes with sugar water and ETO sterilized pollen. Five days post *Crithidia* exposure treatment, bees were snap frozen in liquid nitrogen and stored at -80°C until processing.

**Dissections and DNA extractions**

Prior to dissections, bees were thawed at room temperature. Forewings were removed and the radial cell length was measured as a proxy for body size to be used as a covariate in analysis (Müller et al. 1996; Schmid-Hempel & Schmid-Hempel, 1996). Bees were then surface sterilized in 70% ethanol for 1 minute, followed by 90% ethanol for 1 minute, rinsed with ultrapure water twice, and dried on a kimwipe tissue. Sterilized bees were placed on a single use autoclaved 4x4 cm square of aluminum foil and their mid and hindguts were dissected with sterilized forceps. Individual dissected guts were then be placed into sterile 1.5 mL screw top tubes with a 2.4 mm metal bead, snap frozen in liquid nitrogen, and stored at -80°C until later processing. Forceps and

micro-scissors used in dissections were sterilized using 5% bleach, 70% ethanol, and ultrapure water sequentially between each sample.

200 µL of 1X Tris-buffer saline (TBS) spiked with 1 ng per µL of pJET 1.2 for normalization (deMiranda *et al.* 2021) was added to each gut sample and guts homogenized using a Bead Ruptor (Omni International) on high for 30 seconds. One hundred µL of the gut homogenate was transferred to a sterile 1.5 mL snap top microcentrifuge with 200 µL of gram-positive buffer with lysozyme for DNA extraction by the IBI Bacterial DNA Extraction kit following manufactures protocols. DNA sample quality was verified using a µDrop plate in a MultiSkan GO plate reader (ThermoFisher, Waltham, MA USA).

**Quantification of core gut microbes and *C. bombi***

Targeted qPCR was carried out for total bacterial load, *S. alvi, Gilliamella spp., Lactobacillus spp.*, *S. bombi, C. bombi* and the pJET spike-in to be used to normalize samples for extraction efficiency differences. qPCR took place in a QuantStudio™ 3 Real-Time qPCR machine using specific primers (Table 1). The cycle parameters used were: i) an initiation stage (50°C for 2 minutes followed by 95°C for 2 minutes), ii) an amplification stage (40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute). Although primer specificity has been verified by sequencing, melt curves generated after each run were checked to ensure specificity of amplification. Quantities, based on values derived from standard curves run on each plate, were normalized to the copies per sample using the ratio of measured pJET values to the original starting pJET amount to account for differential DNA extraction efficiencies between samples. Synthetic standards (IDT gBlocks) for each primer product were used to produce the standard curves with

serial dilutions of synthetic standards with a range from $1x10^3$ to $1x10^9$ copies per μL. Therefore, $1x10^3$ copies per μL was used as the threshold for the limit of detection for samples, with values below this considered to be zero. Each DNA sample was run in duplicate, and any duplicates that had a calculated coefficient of variation above 0.20 were rerun (according to Palmer-Young et al. 2018).

**Statistical analysis**

All statistical analyses were performed using R version 4.2.1 "Funny-Looking Kid" for Mac OS. Models were fit with the *glmmTMB* package (Brooks *et al.* 2017). Potential distributions of each response variable were examined for model fit and adherence to model assumptions. The package *emmeans* was used to produce Estimated Marginal Means, with confidence intervals and post-hoc comparisons including Tukey correction for multiple testing (Lenth *et al.* 2020). For all analyses, the original source colony of bees was included as a random effect, and for analyses of total and specific bacterial loads determined by qPCR, the strain of *Crithidia* was also included as a random effect. *Crithidia* infection, with initial fixed effects of body size, *Crithidia* strain, *Crithidia* exposure day and the interaction of strain and exposure day, was analyzed using a Hurdle model, a two-part model that addresses excess zero counts within a dataset. This model utilizes both a zero inflated model to determine the binary likelihood of infection, as well as a conditional model based on the continuous infection level. The conditional model used a negative binomial distribution (*truncated_nbinom2*). Total bacteria and quantities of the specific target bacteria in the gut were analyzed with fixed effects of *Crithidia* treatment, exposure day, their interaction, and body size. Models were compared and simplified using likelihood ratio tests and AIC, and statistics of terms removed from the models were taken from the step before their removal. All

bacterial quantification models were fit with a negative binomial distribution (*nbinom2*) but with the addition of zero-inflation for *Lactobacillus*, *Gilliamella*, and *Snodgrassella.* These models were also fit with data comparing either control versus all *Crithidia* exposed bees or control versus only those *Crithidia* infection positive bees.

# CHAPTER III: RESULTS

Within the *C. bombi* treatment group, there was a significant effect of strain on the proportion of positive infections ($\chi^2 = 17.07$, d.f. = 2, $p < 0.001$, Figure 2A) but not on infection intensity of infected individuals ($\chi^2 = 1.31$, d.f. = 2, $p = 0.518$, Figure 2B)**.** Body size did not significantly affect the proportion positive ($\chi^2 = 0.27$, d.f. = 1, $p = 0.604$) or infection intensity ($\chi^2 = 1.30$, d.f. = 1, $p = 0.254$).

Infection with *C. bombi* significantly reduced the total number of bacteria in the gut (Table 2A, Figure 3). However, this effect was not present when including all *Crithidia* exposed bees, not just those positive for infection (Table 2A). The total bacteria in the gut were also significantly reduced in the late (1,126,034,249, s.e. = 250,439,781) versus the early exposed group (Estimated marginal mean = 1,579,340,044, s.e. = 347,167,064).

Focusing on specific members of the gut microbiota, there is a significant interaction between *Crithidia* treatment and day of exposure for *Lactobacillus* numbers in the gut (Table 2B, Figure 4). There was a reduction following early exposure but not for late exposure, independent of whether exposed or only infected individuals were included in the *Crithidia* treatment group. There was also a significant interaction between *Crithidia* treatment and day of exposure for *Gilliamella* numbers in the gut (Table 2C), with an increase following early exposure or infection but not for late exposure of infection with *C. bombi*, relative to numbers within unexposed individuals (Figure 5). There was no significant effect of *Crithidia* exposure or infection, nor their interaction with exposure day for numbers of *S. bombi* and *S. alvi* in the bumble bee gut (Table 2D and E). However, *Schmidhempelia* numbers were affected by time, with bees within the early

group (5,749,396,640, s.e. = 2,200,765,811) having significantly greater intensities of this bacteria than the later group (3,113,405,116, s.e. = 1,195,471,048) (Table 2D). This same effect of time was seen in *Snodgrassella*, but it was only significant in the group excluding *Crithidia* exposed but uninfected bees. In this case, the bees within the early group (1,234,858,917, s.e. = 632,582,319) again had significantly greater intensities than the later group (605,855,082, s.e. = 315,584,282) (Table 2D). In many cases there was also a significant effect of host body size on bacterial numbers, with this being a positive relationship in all cases (Table 2).

# CHAPTER IV: DISCUSSION

Multipartite interactions between parasitic microbes, hosts, and the host's native gut microbiota form a network where a range of effects varying in their directionality, sign or whether they are direct or indirect can take place (Armitage et al. 2022; Abraham et al. 2017). In bumble bees, exposure to pathogens, including *Crithidia bombi*, have been shown to have a range of negative effects on the host (Cameron & Sadd, 2020). By investigating the effects of infection at two different points of native gut microbiota establishment, we show that infection by *C. bombi* decreases the overall bacteria in the bumble bee gut microbial community in infected individuals when compared to uninfected individuals. Furthermore, specific quantification of core microbiota members shows that exposure to *C. bombi* early in microbiota establishment (day 0) significantly reduced *Lactobacillus spp.* and increases *Gilliamella spp.* in exposed when compared to unexposed workers.

Our results support the hypothesis that infection can disrupt the balance of a native gut microbiota structure. The effect of *C. bombi* infection on the gut microbiota adds to our understanding of the multipartite interactions between these two parties and the bumble bee host. Prior studies have demonstrated a directional effect of the microbiota on *Crithidia* infection, with a native microbiota and specific assemblages of it facilitating host resistance (Mockler et al. 2018; Koch & Schmid-Hempel, 2011). This effect could precipitate associations found in natural populations of bumble bees between particular microbiota structures and the presence of infection by *C. bombi* (Cariveau et al. 2014; Felden et al. 2021). However, the disruptive effect of *Crithidia* infection on the microbiota in increasing *Gilliamella* bacterial load that we show here could also be responsible for such patterns. The positive effect on *Gilliamella* of infection is contrary to the

negative association between this bacterium and the occurrence of infection in one of the field studies (Cariveau et al. 2014). However, given how various sources of heterogeneity can influence infection (Sadd & Barribeau, 2013), it is reasonable to expect that the bidirectional effects between *Crithidia* and the gut microbiota will be context dependent. Some of these can include resource availability (Logan et al. 2005), commercially purchased or wild caught bee origin (Colla et al. 2006), or even bee age (Tobin et al. 2022). In addition, they are likely non-mutually exclusive effects, but rather add to the complexity within this multipartite interaction.

The results also demonstrate that the effect of pathogenic infection on the host's beneficial gut microbiota may also depend upon the timing of infection relative to microbiota establishment. Although total bacteria numbers were reduced in infected versus uninfected bees across the early and late groups, with no interaction between *Crithidia* treatment and exposure time, effects on *Lactobacillus* and *Gilliamella* were only found in those bees exposed early in the establishment of the microbiota. This suggests that the establishing microbiota may be more susceptible to perturbation mediated by infection, than a more established microbiota. In humans, developing microbiota may be more susceptible to perturbations leading to variable structure and function (Vaishampayan et al., 2010). The temporal persistence of dysbiotic effects on the microbiota is worthy of future consideration. For example, recovery from antibiotic perturbation of human gut microbes begins after a week, but recovery is never completed (Dethlefsen and Relman, 2011). Long-term persistence of changes to microbiota structure and function that may even last beyond the removal of the responsible external stimulus could be considered more detrimental. Thus, the persistence of the effect of *Crithidia* exposure on the microbiota will have important consequences for understanding the severity of the imbalance for hosts.

The demonstrated effects of *C. bombi* on the bumble bee microbiota could come about through many different mechanisms, direct or indirect, as outlined in the introduction (Armitage et al. 2022). *Crithidia* cells infect within the hindgut of the host and attach via the flagella to the gut epithelium (Koch et al. 2019). Thus, they could directly affect the members of the gut microbiota through exploitation competition for shared space or nutrition or through antagonistic interference competition. Particular bee gut microbes breakdown pectin in pollen or use subsequent breakdown products (Engel et al. 2012; Zheng et al. 2016). *Crithidia* infection intensity is positively affected by pollen availability (Logan et al. 2005), suggesting that it may be a limiting resource, and a resource for which infecting *Crithidia* may compete with native gut microbes. An alternative to the direct effects outlined above are indirect effects mediated through the host. For example, the infection by *Crithidia* leads to changes in host immune gene expression, including antimicrobial peptides (Barribeau & Schmid-Hempel, 2013; Barribeau et al., 2014). Induction of the host immune response could perturb its own microbiota. Alternatively, if host immunity is acting to keep the microbiota in a balanced state, as is the case in other systems (Bosco-Drayon et al. 2012; Nyholm & Graf, 2012), disruption of immunity could lead to changes in the microbiota structure. The different direction of effects of *Crithidia* infection on *Lactobacillus* and *Gilliamella* suggest that only competition for space is unlikely, and that whatever the driver its effects are specific to particular gut microbiota members.

The beneficial effects of a native healthy gut microbiota in bumble bees and honey bees (e.g., Koch & Schmid-Hempel, 2011; Zheng et al. 2016; Kwong et al. 2017; Zheng et al. 2017), suggests the alteration of the bacterial gut community by *Crithidia* exposure will likely have

functional consequences. In fact, it is possible that these changes to the gut microbiota due to infection could underly already described pathological effects on the host. For example, infection affects longevity and reproduction in workers and queens (Brown et al., 2000; Brown et al. 2003; Fauser et al. 2017). It is possible that some documented effects come from specific changes, rather than overall disruption, such as the reduction in *Lactobacillus.* In honey bees, *Lactobacillus* is considered to be an important part of the gut-brain axis, where it promotes learning and memory behavior through regulation of tryptophan metabolism (Zhang et al. 2022). Thus, a reduction in *Lactobacillus* could mediate the reduction in learning, foraging and flower handling ability following *Crithidia* infection (Gegear et al. 2005; Gegear et al. 2006).

Overall, this work adds to the increasing appreciation of the multi-dimensionality and directionality of effects in multipartite networks of hosts, their beneficial microbes, and pathogens. This signals the need to include all these parties when considering the factors determining the occurrence of infection and outcomes when infection occurs. Ultimately, depending on exact community structure and functional effects, these interactions will have consequences for host health but also ecological and knock-on evolutionary effects for hosts and their associated microbes, wherever they lie on the spectrum from beneficial to detrimental.

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**Table 1.** The qPCR primers used to quantify *C. bombi* infection, total bacteria, specific host core gut microbe targets, and a spike-in control used to account for differences in extraction efficiencies.

| Target | Primer sequences (5' to 3') | Reference |
|---|---|---|
| *Snodgrassella alvi* | **F:** CTTAGAGATAGGAGAGTGCCTT<br>**R:** AACTTAATGATGGCAACTAATGACAA | Kešnerová *et al.*, 2017 |
| *Gilliamella spp.* | **F:** CTTTGTTGCCATCGGTTAGGCC<br>**R:** CCGCTTGCTCTCGCGAGG | Kešnerová *et al.*, 2017 |
| *Lactobacillus spp.* | **F:** CGTGCCTAATACATGCAAGTCG<br>**R:** GGCTAAAGGGTAGGTTGCCC | Sauers *et al.* in prep |
| *Schmidhempelia bombi* | **F:** CTGGTCGTCTGGAGTATTGT<br>**R:** AGGTCCGCTCACCATCGCTG | Martinson et al. 2014 |
| *Crithidia bombi* | **F:** GGCCACCCACGGGAATAT<br>**R:** CAAAGCTTTCGCGTGAAGAAA | Ulrich *et al*., 2011 |
| Universal | **F:** AGGATTAGATACCCTGGTAGTCC<br>**R:** YCGTACTCCCCAGGCGG | Kešnerová *et al.*, 2017 |
| pJET | **F:** CGAGGTTTAGAGCAAGCTTC<br>**R:** ACACTTTATGCTTCCGGCTC | de Miranda *et al.,* 2021 |

**Table 2.** Model terms and statistics from generalized linear mixed models with negative binomial error distributions, in some cases with zero inflation, fit to data from the quantification of total and specific bumble bee gut bacteria.

| **Model term** | **All data** | | | **Control versus *Crithidia* infected** | | |
|---|---|---|---|---|---|---|
| **A) Total bacteria** | $\chi^2$ | ***d.f*** | ***P*** | $\chi^2$ | ***d.f*** | ***P*** |
| Body size | 4.42 | 1 | **0.036** | 1.29 | 1 | **0.256** |
| Exposure day | 4.71 | 1 | **0.030** | 2.31 | 1 | **0.128** |
| *Crithidia* treatment | 0.23 | 1 | 0.635 | 6.92 | 1 | **0.009** |
| Exposure day x *Crithidia* treatment | 0.99 | 1 | 0.319 | 0.74 | 1 | 0.390 |
| **B) *Lactobacillus*[a]** | | | | | | |
| Body size | 18.46 | 1 | **<0.001** | 3.45 | 1 | **0.06** |
| Exposure day | 6.70 | 1 | **0.019** | 9.08 | 1 | **0.003** |
| *Crithidia* treatment | 1.60 | 1 | **0.195** | 17.17 | 1 | **<0.001** |
| Exposure day x *Crithidia* treatment | 4.41 | 1 | **0.035** | 4.52 | 1 | **0.033** |
| **C) *Gilliamella*[a]** | | | | | | |
| Body size | 24.08 | 1 | **<0.001** | 22.17 | 1 | **<0.001** |
| Exposure day | 5.32 | 1 | **0.021** | 5.38 | 1 | **0.020** |
| *Crithidia* treatment | 10.39 | 1 | **0.001** | 10.42 | 1 | **0.001** |
| Exposure day x *Crithidia* treatment | 5.29 | 1 | **0.022** | 9.10 | 1 | **0.003** |
| **D) *Schmidhempelia*** | | | | | | |
| Body size | 9.06 | 1 | **0.003** | 2.52 | 1 | **0.113** |
| Exposure day | 10.18 | 1 | **0.001** | 11.04 | 1 | **<0.001** |
| *Crithidia* treatment | 0.17 | 1 | 0.678 | 2.00 | 1 | 0.157 |
| Exposure day x *Crithidia* treatment | 0.09 | 1 | 0.764 | 0.75 | 1 | 0.386 |
| **E) *Snodgrassella*[a]** | | | | | | |
| Body size | 40.49 | 1 | <0.001 | 19.90 | 1 | <0.001 |
| Exposure day | 1.62 | 1 | 0.203 | 7.57 | 1 | 0.006 |
| *Crithidia* treatment | 0.02 | 1 | 0.878 | 0.00 | 1 | 0.994 |
| Exposure day x *Crithidia* treatment | 0.54 | 1 | 0.462 | 0.39 | 1 | 0.535 |

Note: Bold terms represent terms in the final best models, with statistics of the other terms taken from before their removal. [a] With zero-inflation, but zero-inflation terms are not presented as final models only included the intercept in the zero-inflation component.

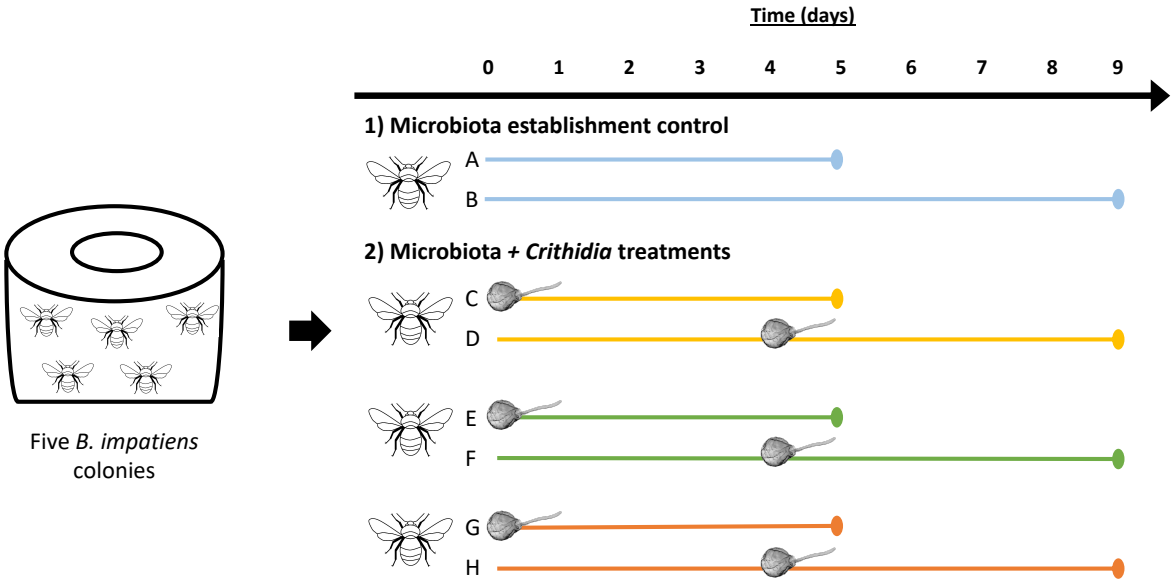
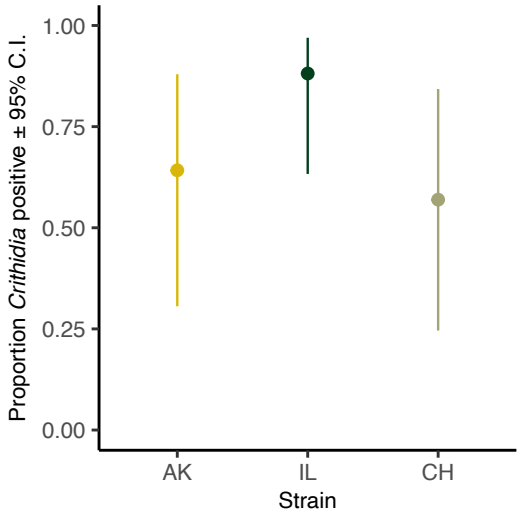



**Figure 1. Schematic of the overall experimental design.** Newly emerged workers were given their microbiota inoculum and placed into one of eight treatments (combinations of timing and type of pathogen exposure (A. Early naïve, B. Late naïve, C. Early strain 1, D. Late strain 1, E. Early strain 2, F. Late strain 2, G. Early strain 3, H. Late strain 3). Five days after pathogen exposure treatment, bees were snap frozen in liquid nitrogen before their guts were dissected for quantification of the total bacteria, numbers of key bumble bee gut microbes, and *C. bombi*.

A.




B.

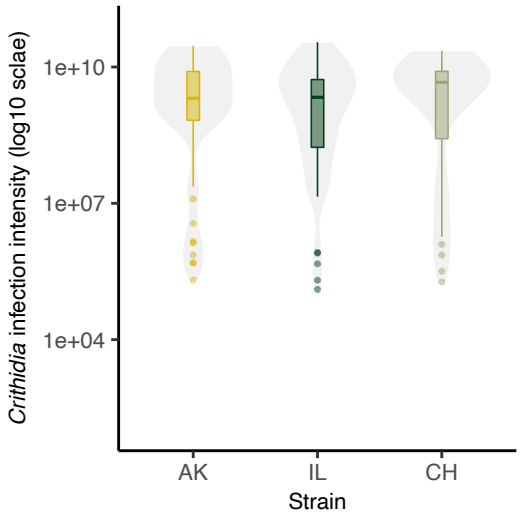



**Figure 2. *Crithidia bombi* infection across strains.** A) Proportion of *Crithidia* positive individuals (estimated marginal means with 95% confidence intervals). B) Violin plots with nested boxplots showing *Crithidia* infection intensities in *Crithidia* positive individuals. Dark horizontal lines within each box indicates the median, the box the interquartile range, and the whiskers the upper and lower values. The gray-shaded violin shapes indicate the distribution of the data, with wider portions indicate a higher sample density.

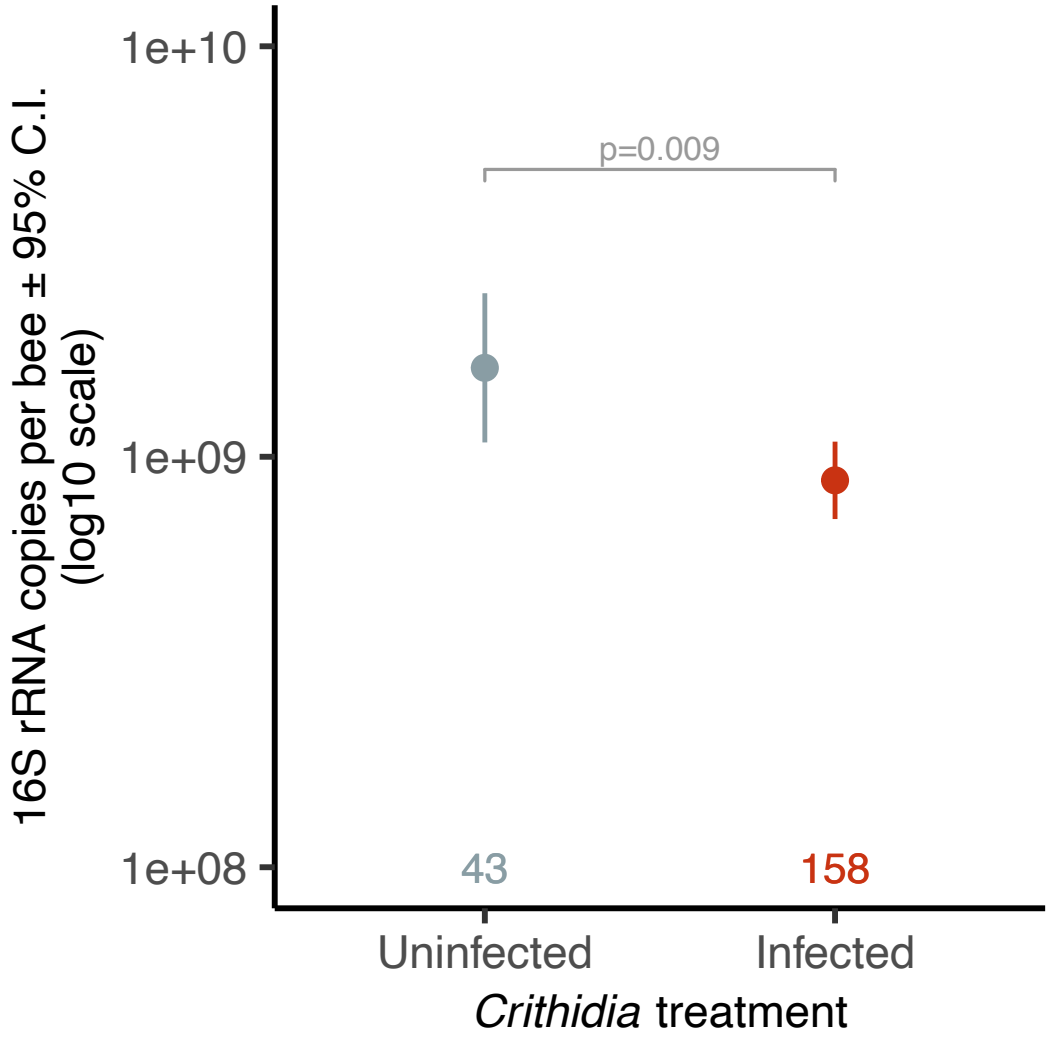



**Figure 3. Total bumble bee gut bacteria depending on *Crithidia* infection.** Points represent the estimated marginal means, and bars represent 95% confidence intervals. Sample size is noted along the X axis.

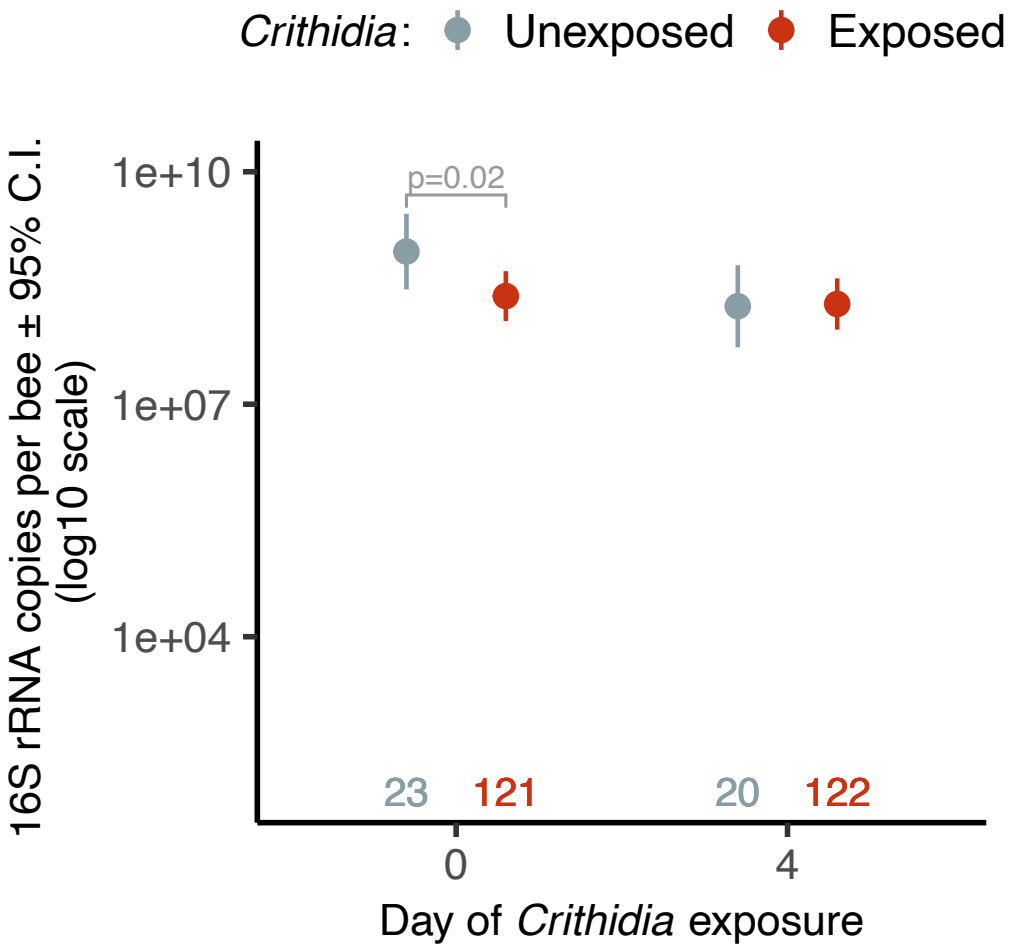



**Figure 4. The influence of *Crithidia* exposure treatment and day on *Lactobacillus spp.* quantity in the bumble bee gut.** Points represent the estimated marginal means, and bars represent 95% confidence intervals. Sample size is noted along the X axis.

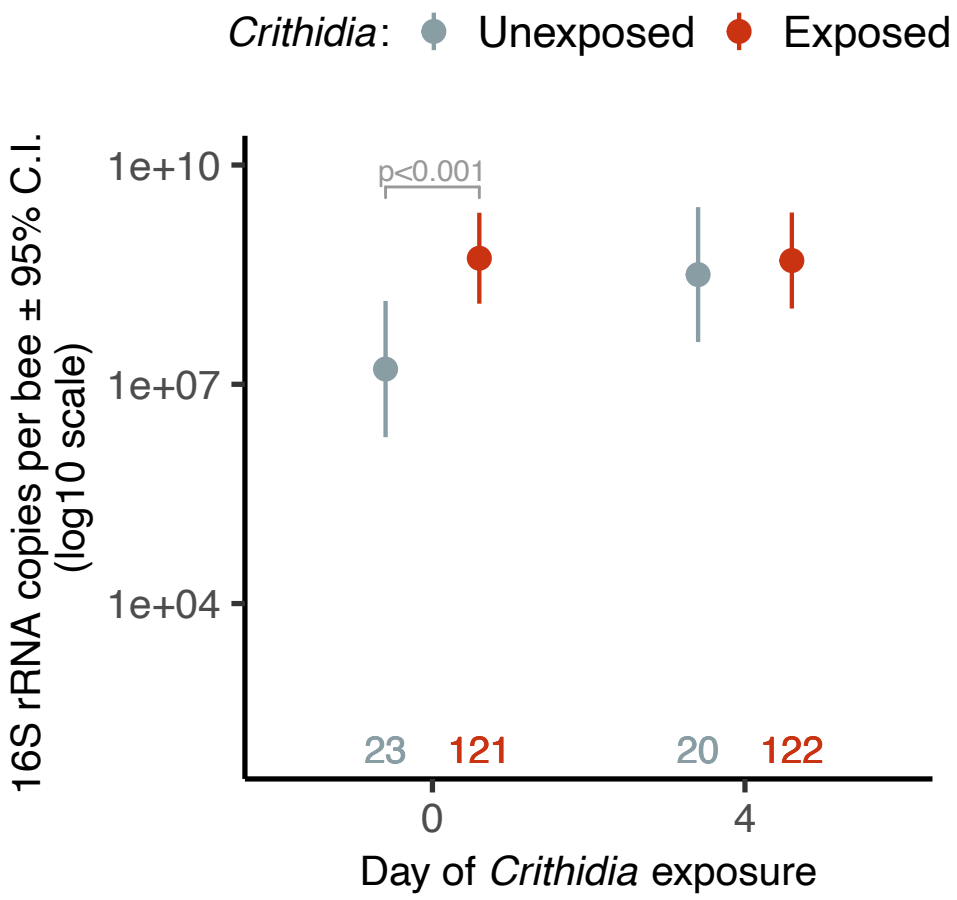



**Figure 5. The influence of *Crithidia* exposure treatment and day on *Gilliamella spp.* quantity in the bumble bee gut.** Points represent the estimated marginal means, and bars represent 95% confidence intervals. Sample size is noted along the X axis.