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Consequences of Microsporidian Prior Exposure for Virus Infection Outcomes and Bumble Bee Host Health

Elyse Christine McCormick
Illinois State University, emccorm1@iwu.edu

CONSEQUENCES OF MICROSPORIDIAN PRIOR EXPOSURE FOR VIRUS INFECTION OUTCOMES AND BUMBLE BEE HOST HEALTH

ELYSE CHRISTINE MCCORMICK

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Host-parasite interactions do not occur in a vacuum, but in connected multi-parasite networks. Resulting co-exposures and coinfections during an individual host's lifetime can affect host health and infectious disease ecology, including disease outbreaks. However, many host-parasite studies examine pairwise interactions, meaning we still lack a general understanding of the influence of co-exposures and coinfections. Using the bumble bee *Bombus impatiens*, we study the effects of larval exposure to a microsporidian *Nosema bombi*, implicated in bumble bee declines, and adult exposure to Israeli Acute Paralysis Virus (IAPV), an emerging infectious disease from honey bee parasite spillover. We hypothesize that infection outcomes will be modified by co-exposure or coinfection depending on relevant temporal interactions, due to changes in host immune allocation or condition. *Nosema bombi* is a potentially severe, larval-infecting parasite, and we predict that prior exposure will result in decreased host resistance to adult IAPV infection. We predict a double exposure will also reduce host tolerance, as measured by host survival. Although our larval *Nosema* exposure mostly did not result in viable infections, it reduced resistance to adult IAPV infection. Exposure to *Nosema* also negatively affected survival, potentially due to a cost of immunity in resisting the exposure. There was also a significant negative effect of IAPV exposure on survivorship, but in contrast to resistance, prior *Nosema* exposure did not alter this survival outcome. These results again demonstrate that

infection outcomes within multi-parasite host networks can be non-independent, even when exposure to one parasite does not result in a substantial infection.

KEYWORDS: bumble bee, coinfection, co-exposure, host-parasite interactions, *Nosema bombi*, IAPV, pollinator

CONSEQUENCES OF MICROSPORIDIAN PRIOR EXPOSURE FOR VIRUS INFECTION OUTCOMES AND BUMBLE BEE HOST HEALTH

ELYSE CHRISTINE MCCORMICK

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ELYSE CHRISTINE MCCORMICK

COMMITTEE MEMBERS:

Ben Sadd, Chair

Adam Dolezal

Rachel Bowden

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CONTENTS

CHAPTER I: INTRODUCTION

Host-parasite interactions do not occur in a simple two player network, but rather in communities with a connected network of multiple hosts and multiple parasites [1]. In individual hosts, this means co-exposures or coinfections can occur during an individual host's life, through simultaneous or sequential parasite encounters. Yet, many studies in model systems for understanding evolutionary and ecological disease dynamics and infection outcomes focus on host-parasite pairwise interactions. Model pairwise systems such as house finches (*Carpodacus mexicanus*) and their conjunctivitis-causing parasite *Mycoplasma gallisepticum* [2], monarch butterflies (*Danaus plexippus*) and the neogregarine *Ophryocystis elektroscirrha* [3], and bumble bees (*Bombus* spp.) and the gut trypanosome *Crithidia bombi* [4] have provided significant advances in our knowledge in host-parasite interactions. However, while pairwise infection studies are important and informative, they may miss complex and key interactions and outcomes that stem from co-exposures and coinfections in natural systems.

Coinfections are common in nature, ranging across host taxa and the relatedness of the infecting agents, from multiple anther smut strains (*Microbotryum violaceum*) infecting the flower *Silene latifolia* [5], to coinfections of *Brucella* spp. bacteria and Cetacean Morbilivirus in sperm whales (*Physeter macrocephalus*) [6], and helminths and malaria in humans [7,8]. In these and other systems, the interdependence of infection outcomes during coinfection can lead to changes in parasite transmission and virulence relative to single infections [1,9]. Due to the complexity of these interactions, coinfections are likely to have wide-reaching consequences for the health of hosts, parasite disease ecology, and host-parasite evolution [10–13]. Although infection outcomes may not be modified in all cases of co- versus single infections [14], variation in coinfection dynamics makes studying co-exposures and coinfections resulting from

multi-parasite networks an central open topic in disease ecology [15]. This is particularly relevant given ongoing global shifts through climate change and anthropogenic disturbance that has the potential to bring hosts into contact with novel combinations of parasites and other stressors [16,17]. Indeed, the contact with novel parasites is exemplified by the SARS-CoV-2 pandemic in humans [18], indicating the importance of understanding disease dynamics and infection outcomes.

The outcomes of coinfection for hosts and parasites can vary, and depend on the biology of the interacting parties and both direct and indirect interactions between infecting parasites, such as space and resource use or changes in host-mediated immunity [19–21]. In the water flea *Daphnia magna*, bacterial and microsporidian coinfection had detrimental consequences for the host and both parasites [22], and in a snail (*Biomphalaria glabrata*) co-exposure to the trematodes *Schistosoma mansoni* and *Echinostoma caproni* resulted in increased virulence and exploitation of the host by the latter [23]. Whether such co-exposures are simultaneous or sequential and the order of sequential exposures can also determine infection outcomes [24]. For example, success of the trematode *Ribeiroia ondatrae* infecting the Pacific chorus frog was reduced by prior *Echinostoma trivolvis* infection, but there was no effect when the order was reversed [25]. The outcomes of coinfection may also depend on the environment in which they take place, with global change potentially exacerbating negative effects. Serengeti lions (*Pathera leo*) show higher mortality from coinfections of canine distemper virus (CDV) and tick-borne apicomplexan *Babesia* spp. during times of drought than they would in non-drought conditions [26].

As outlined above the underlying mechanisms that determine differential outcomes under a scenario of co-exposure or coinfection will vary between the hosts and their parasites that are

involved, but focusing on host immunity, responses when parasites are experienced simultaneously or sequentially may differ from responses to exposure to those parasites in isolation. A meta-analysis on coinfection in mice revealed that the direction of coinfection effects of helminths on micro-parasites varies depending on underlying mechanisms of infection, including host immunity [21]. The immune system has associated evolutionary and usage costs, with physiological and resource based trade-offs resulting in intrinsic compromises when mounting and maintaining an immune response [27,28]. For example, internal trade-offs between specific arms of the immune system [20,29] may lead to sub-optimal responses by the host when responding to multiple parasites. Additionally, immune suppression by one parasite can also facilitate another, as is the case for invasion of bovine tuberculosis being facilitated by prior nematode-induced immune suppression in African Buffalo (*Syncerus caffer*) [30]. Thus, even a relatively benign parasite that elicits a costly immune response or suppresses immunity may have serious consequences if this occurs concurrently with an infection of a potentially more severe parasite. Even when a parasite exposure does not lead to a viable lasting infection, it can have cascading effects on future host defense and infection outcomes. For example, immune priming in invertebrates can result in enhanced protection to future parasite exposures following a previous non-lethal infection or parasite exposure [31–35]. While priming can be specific to the parasite type [36], in other cases general immune priming responses can provide broad protection [37]. The balance of the costs of immunity, immune suppression and immune priming, whether general or specific, will have implications for systems where hosts experience sequential time-lagged co-exposures or coinfections.

Despite the importance of coinfection in nature, in many systems where understanding infectious disease outcomes is of relevant for such areas as conservation or pest control, we still

lack an understanding of the effects of coinfection on infection outcomes and host-parasite dynamics. Though such a knowledge gap is recognized [1,15], coinfection outcomes are rarely studied [4,38]. This is true in bees, which are ideal model systems for host-parasite studies due to their practicality for study, their myriad of ecological merits, and the potential significance of parasites in bee population and community health and conservation [39]. Bumble bees (*Bombus* spp.), wild and managed pollinators of ecological, economic, and conservation concern, are exposed to multiple parasites in a multi-species plant-pollinator network [40]. Infections, infection intensity, and infection outcomes can vary in response to the surrounding environment, stressors such as poor nutrition and pesticide exposure, and interactions in the larger bee community [41–47].

A key bumble bee parasite, also touted to be associated with the declines of some bumble bee species, is the microsporidian *Nosema bombi* [48,49]. This parasite predominantly infects at the larval stage, where, following ingestion of environmentally resistant spores, it ejects a coiled polar filament infecting cells in the gut [50–52]. The parasite is subsequently found in multiple tissues, including the Malpighian tubules, thorax muscles, fat body tissue and nerve tissue, and the brain [53], causing considerable tissue damage for individual bees. Additionally, as a microsporidian, *N. bombi* manipulates the host's mitochondrial machinery to hijack energy [54,55], which will drain host energy stores and could cause physiological problems [56]. *Nosema bombi* infection has a number of fitness-relevant consequences for bumble bees, including reducing queen colony founding success [57], male and worker longevity in the laboratory [58], and the size of field colonies [59]. Its high virulence and the fact that its prevalence of infection is highest in declining North American bumble bee species [48,49], make studying the context dependence of infection outcomes highly relevant. Despite *N. bombi* being

nested in a community of bumble bee parasites [40], we currently have limited information on how coinfection affects bumble bees or its transmission.

Wild bumble bee parasite communities can be influenced by the composition of the host communities, including the presence of managed pollinators such as honey bees and commercial bumble bees [41,60,61]. Recently, spillover of viruses, in which viral parasites prevalent in honey bees are transmitted to bumble bees and other native bees, has gained increasing attention [47,61–67]. Spillover may create problems for the native bumble bee community, altering the risk and identity of infections [68]. For example, Israeli Acute Paralysis Virus (IAPV) has been found at high levels in bumble bees close to infected honey bee apiaries [67]. IAPV, a positive-sense, single-stranded RNA virus in the Picornavirus order and *Dicistroviridae* family, is predominantly associated with honey bees, in which the accumulation of virus particles and the suppression of essential cellular components results in host cell death [69]. IAPV invades almost all honey bee tissues, causing decreased motor function, severe muscle spasms, paralysis, and death [70,71]. Though IAPV can affect all honey bee life stages, it has significant impacts on adult gene expression, and can replicate to higher levels than other bee viruses when in a mixed inoculum [70,72]. Experimental infections of IAPV in bumble bees result in foreleg paralysis, decreased desire to consume nectar, apathy to disturbance, lethargy, severe muscle spasms, and increased mortality, as well as similar virus tissue tropism to honey bees [73,74]. In adult bumble bees IAPV inoculation triggers the RNAi antiviral immune response [74], which is costly for the bee. The combination of these effects indicates that IAPV is potentially a substantial threat to bumble bee health when infecting alone. However, no studies have been carried out to determine how its presence in bumble bee communities may interact with existing host-parasite relationships.

Viral effects on bumble bees suggest that honey bee-derived viruses, like IAPV, may interact with other bumble bee parasites to alter the outcomes of infection. In the only experimental coinfection study in bumble bees to date, Graystock *et al.* noted that coinfection of the neogregarine *Apicystis bombi* and the predominantly honey bee Deformed Wing Virus (DWV) altered both lethal and sub-lethal effects of infection, increasing individual mortality up to 50%, reducing fat bodies by 17%, and increasing sensitivity to sucrose [38]. Further studies are required to assess the potential consequences of parasite spillover from managed to native wild bees resulting in increased novel coexposures and coinfections of parasites. Our work presented here investigates how sequential exposures of *N. bombi* and IAPV influence infection outcomes in the bumble bee *Bombus impatiens*. The overarching hypothesis is that, due to changes in host allocation to immunity, infection outcomes of *N. bombi* and IAPV will be modified by sequential co-exposures and coinfection, with consequences for host-parasite dynamics, including host resistance and tolerance and parasite virulence and transmission. Resistance is defined as the ability to limit the infectious burden, while tolerance is the ability to mitigate the health impact associated with a given parasite infection intensity [75]. Given the time-lagged nature of exposure to *N. bombi* in larvae and IAPV in adults, we predict that prior *Nosema* exposure will lead to decreased resistance to IAPV. In addition, tolerance is predicted to be affected, with co-exposure and coinfection reducing host survival more than single infections.

CHAPTER II: MATERIALS AND METHODS

Overall experimental design

Experimental coinfections were performed to evaluate how time lagged exposures of *N. bombi* and IAPV in *B. impatiens* influences infection outcomes. To address these objectives, microcolonies were established from laboratory reared bumble bee colonies. Larvae in these microcolonies were fed *N. bombi* or not, and subsequently adults were injected with IAPV or not, thus creating a fully reciprocal crossed design of four exposure treatments: 1) co-exposed to *Nosema bombi* and IAPV, 2) *Nosema* exposed, 3) IAPV exposed, or 4) unexposed (Figure 1). Subsequently, parameters relating to host tolerance (survival) and resistance (infection loads), and parasite transmission (spores) were assessed.

Bumble bees and parasites

Bombus impatiens queens of lab-reared colonies were collected with the permission of the ParkLands Foundation (http://www.parklandsfoundation.org/) from the Mackinaw River Study Area (Lexington, IL., U.S.A.). These bees were reared at 26 ± 2°C, with red-light illumination, fed pollen (Swarmbustin' Honey, Chester County, Pennsylvania, United States) three times per week, and given sugar water (ratio 1 g granulated white sugar, 1 mL boiled tap water, 0.1% cream of tartar) *ad libitum*. Visual and molecular screens of the queen and subsequently produced workers for common parasites, including *Nosema bombi* and *Crithidia bombi*, were performed by obtaining and observing fecal samples under phase contrast microscopy (400x total magnification) and performing diagnostic PCR to ensure that colonies were healthy and initially pathogen-free.

Nosema bombi spores from strain VT21.46, sourced from worker bees of a naturally infected *Bombus terricola* queen bumble bee from Vermont, United States, were used (Vermont Agency of Natural Resources permit ER-2021-13). Spores were prepared as in [76] , quantified, and were stored at -80℃ until use in experimental inoculation.

IAPV was extracted from homogenized honey bee pupae using an established protocol [72,77]. An enriched mixture of virus particles was stored at -80℃ until use in experimental inoculations. A preliminary dose-dependence study of survival to IAPV was performed to determine an infective dose to use. Inoculums were serially diluted from the original viral stock with PBS to produce dilutions to between 10^{-8} and 10^{-12} of the original stock. Both adult males and workers of *B. impatiens* were injected with this range of doses, or sham inoculated. Based on the outcome of these preliminary trials, a dilution of $10^{-8.5}$ from the original stock was chosen as an effective dose for initiating infection, with mortality in 75% of bees between five and ten days after inoculation.

Microcolony preparation and *Nosema* inoculation

Original source colonies were monitored until second-instar larval brood were present in the colony. This brood was then carefully isolated from the source colony, and maintained as a queenless microcolony with three marked adult workers. Microcolonies allow genetic background to be controlled across treatments, for robust sample size and replication [78]. Original colonies were kept, providing bees to maintain the attending adult worker population throughout microcolony development, and to begin new microcolonies when appropriate brood became available. Microcolonies from within a source colony of origin were randomly assigned to one of two *Nosema* treatments: *Nosema*-exposed or *Nosema*-free. After 24 hours of

microcolony acclimation, each larva within *Nosema*-exposed microcolonies was individually inoculated with 40,000 *N. bombi* spores in 2µl of a sugar water and pollen solution [76]. Larvae of *Nosema*-free microcolonies were given a parasite-free solution by the same method. The microcolonies were observed daily for adult emergence, and emerging adult bees were isolated in individual deli containers (13.5 x 10 x 5.7 cm) with *ad libitum* pollen and sugar water, and randomly assigned to an IAPV treatment, creating all possible combinations of *N. bombi* and IAPV exposure.

IAPV injections

Three days post adult emergence bees were placed in vials and anesthetized on ice for approximately 15 minutes. Once anesthetized, bees were injected between the first and second abdominal tergite with 2 µl of either their pre-assigned viral inoculum (at $10^{-8.5}$ of the original stock in PBS) or a sham injection of 2 µl PBS using a pulled glass capillary tube to inject. Bees were then allowed to recover at room temperature and were again placed in their individual holding boxes with *ad libitum* sugar water and pollen. While the typical transmission route of bee viruses may be a fecal-oral route [79], injections provide practical benefits, such as improved visibility and robustness of infection for controlled experimental inoculations [73]. It is also plausible that injection could mimic a vectored virus. Although there are no records of IAPV as a vectored virus in bumble bees to date, there are parasitic flies that attack both honey bees and bumble bees which have been shown to carry DWV [73,80,81]. In addition, parasitic mites of honey bees, such as *Varroa destructor*, act as a virus vector [82,83]. Although, there is as of yet no documented role of mite vectored viruses in bumble bees, bumble bee infecting tracheal mites, such as *Locustacarus buchneri* [84,85], carry such a potential.

Infection outcomes: survival and infection prevalence and intensities

Survival was monitored daily. A random subset of bees was sacrificed at 4 days post IAPV inoculation (7 days post adult eclosion) for a time-controlled determination of infection intensities. The remaining bees continued to be checked for survival for at least 15 days post IAPV inoculation (18 days post adult eclosion). This time is a close representation of the lifespan of an average foraging bumble bee worker in nature [86]. If an individual in the experiment survived past this time, they were frozen for other measurements and treated as censored in the survival analysis. Body size was measured for all individuals based on the body size surrogate of the length of the radial forewing cell [87,88], using ImageJ software on images taken with a microscope-mounted camera. Both forewings were measured, and the average used for subsequent analyses.

To prepare samples for molecular quantification of infection levels, samples were homogenized and spike-in nucleic acid references of both DNA and RNA were added to the buffer before extraction, adapted from methods in de Miranda *et al.* [89]. Bee abdomens were removed on ice and added to 800 µl of a 1.5 ml screwcap tube containing a buffer mix of TBS, RNA250 (10 ng/ul), and pJET1.2 Cloning Vector (1 ng/ul), with three 2.4mm steel beads in a 1.5 ml screwcap tube. RNA250 and pJET1.2 are synthetic, passive nucleic acid reference standards that were added into the buffer mixture at standard quantities to allow for correction of differential extraction efficiencies between samples. The resulting homogenate was split into 250 ul for RNA extraction, 250 ul volume for *Nosema* DNA extraction, and the remainder for microscopy. For RNA extraction, 750 ul of TRIzol LS was added and samples placed in a -80°C

freezer until further extraction. The homogenate for DNA extraction and microscopy were placed in a -20°C freezer.

To extract IAPV RNA from the samples, each homogenate with TRIzol LS was removed from -80ºC and incubated at room temperature for 15 minutes. Next, 900 µl of the homogenate mixture was transferred to a fresh tube containing 100 µl of 1-bromo-3-chloropropane and mixed well by shaking for 10 seconds. Samples were then incubated at room temperature for 5 minutes, before centrifugation at 4°C for 10 minutes at 12,000 g. The aqueous phase was transferred to an RNase-free vial containing 450 µl of 99% isopropanol, and mixed well via aspiration with a pipette. Samples were incubated at room temperature for 7 minutes, and then centrifuged for 10 minutes at 12,000 g. The supernatant was discarded and 500 µl of 75% EtOH (made with nuclease-free water) was added to the samples and aspirated up and down using a pipet until the pellet became loose. These samples were centrifuged for 5 minutes at 12,000 g. The rinse with 75% EtOH was repeated, and the supernatant was discarded, with pellets allowed to air dry for 3-10 minutes. The RNA sample remaining in the tube was re-suspended in 50 µl of nuclease-free water and placed on ice while the RNA quality (260/280 and 260/230 absorbance ratios) and concentrations were checked for quality using a MultiSkan GO (ThermoScientific™) spectrophotometer.

Quantification of IAPV and RNA250 in samples used a one-step RT-qPCR method using a BioRad™ iTaq™ Universal SYBR® One-Step RT-qPCR Kit and a QuantStudio 3 Real-Time qPCR machine (Applied Biosystems™). For IAPV, primers established in Carrillo-Tripp *et al.* were used (forward: 5'-GCACAGTCTTCTGGTGATTGC-3', reverse: 5'-GTTAGCACACGATTGGTTATCAGC-3') [72,90]. Reverse transcription took place at 50°C for 25 minutes, then an initial denaturation step of 95C for 5 minutes, followed by 40

amplification cycles of 15 s denaturation at 95°C and a simultaneous annealing and extension at 58°C. The melting curve was 95°C for 30 seconds and 55°C for 30 seconds with stepwise increases of 0.5C from 55-95°C, as detailed in Carrillo-Tripp *et al.* [72]. RNA250 quantification used primers from de Miranda *et al.* with initial denaturation of 10 minutes at 95°C, followed by 40 amplification cycles of 15 s denaturation at 95°C and a simultaneous annealing and extension at 58°C [89]. Each qPCR plate contained a synthetic standard serial dilution curve of an Integrated DNA Technologies™ gBlock of the target product sequence.

For *Nosema* quantification, DNA was extracted from the sample homogenate according to the manufacturer's protocol using the IBI Scientific™ Fecal DNA Extraction Kit. For each sample, DNA quality (260/280 and 260/230 absorbance ratios) and concentrations were checked using a MultiSkan GO spectrophotometer. qPCR reactions used the Applied Biosystems™ PowerUp™ SYBR® Green Master Mix (300 nM) with established *N. bombi* specific BOMBICAR primers (10 μM each, forward: 5′-GGCCCATGCATGTTTTTGAAGATTATTAT-3′, reverse: 5′-CTACACTTTAACGTAGTTATCTGCGG-3′) [91]. Initial denaturation took place for 10 minutes at 95°C, followed by 40 amplification cycles of 15 s denaturation at 95°C and a simultaneous annealing and extension at 58°C [92]. An additional qPCR plate quantifying the passive nucleic acid reference standard pJET1.2 was also performed [89]. Each qPCR plate again contained a synthetic standard serial dilution curve of an Integrated DNA Technologies™ gBlock of the target product sequence.

Each DNA sample was run in duplicate, and any duplicates that had a calculated coefficient of variation above 0.20 were rerun. Based on the limits of amplification of the synthetic standards, a limit of detection of 150 copies per μl was set, with any samples below this

point classified as zero. Any samples over this threshold were several magnitudes above. Genome copies of IAPV and *Nosema* per sample were calculated from on the per µl quantities of each based on the synthetic standard curve produced using gBlock gene fragments (Integrated DNA Technologies, Coralville, IA, USA). These per µl values were multiplied by the ratio of the measured qPCR quantity of the respective spike-in for each sample (RNA250 for IAPV and pJet for *Nosema*) to the copy number of the spike-in added to the original sample. This gave genome copies of IAPV and *Nosema* per sample that were corrected for any differential extraction efficiencies between samples.

The remaining homogenate was used for phase contrast microscopy at 400x to detect the presence of *Nosema* spores. 10 µl of the homogenate was placed into a FastRead 102 counting chamber and any transmission ready spores were counted.

Data analysis

All statistical analyses were performed using R version 4.1.3 "One Push-Up" for Windows. Linear Mixed and Hurdle models were fit with the *lme4* package [93] and *glmmTMB* package [94], respectively. For survival, Mixed Effects Cox Proportional Hazards models were fit using the package *coxme* [95]. 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 FDR correction for multiple testing [96]. For all analyses, microcolony nested within the original source colony was included as a random effect. For body size, *Nosema* exposure was included as a fixed effect in a linear mixed model with the response variable square transformed. IAPV infection, with fixed effects of *Nosema* exposure and body size, 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*). For survival, fixed effects were *Nosema* exposure, IAPV exposure, and 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.

CHAPTER III: RESULTS

A total of 61 adult workers were assessed for infections in the quantification experiment and 157 adult workers in the survival experiment. For the quantification experiment, these bees came from 13 *Nosema* exposed and 12 unexposed microcolonies set up from eight original source colonies. For the survival experiment, these bees came from 16 *Nosema* exposed and 14 unexposed microcolonies set up from seven original source colonies.

Nosema exposure during development and adult size

There was no significant effect of larval exposure to *Nosema* on adult size ($F = 0.929$, d.f. = 1, 22.43, $p = 0.345$). Mean radial cell lengths, a surrogate for body size, were 2.55mm (s.e. = 0.025mm, n = 99) for unexposed bees and 2.48mm (s.e. = 0.031mm, n = 119) for bees that had been exposed to *Nosema* as larvae.

Infection outcomes of *Nosema* exposed, IAPV exposed and co-exposed bees

Based on qPCR, only one bee of 32 screened from the quantification experiment for *Nosema* was deemed to have an active infection above the limit of detection, with an estimated 1,850,672 *Nosema* genome copies in total. In addition, all samples were screened microscopically for the presence of *Nosema* extracellular spores, with spores only detected in the aforementioned sample. Therefore, subsequently, we only refer to effects of *Nosema* exposure on IAPV infection and survival.

Positive IAPV infections were found in 61.29% of the quantification experiment bees exposed to IAPV. Neither *Nosema* prior exposure ($\chi^2 = 0.140$, d.f. = 1, $p = 0.708$, Figure 2A) nor body size ($\chi^2 = 0.576$, d.f. = 1, $p = 0.448$) significantly influenced if bees showed IAPV positive

infections. Body size also did not significantly affect IAPV infection intensities ($\chi^2 = 0.300$, d.f. = 1, $p = 0.585$). However, prior exposure of bees to *Nosema* had a significant effect on IAPV levels in infected bees ($\chi^2 = 41.27$, d.f. = 1, $p < 0.0001$). IAPV infection levels in bees that had been exposed to *Nosema* as larvae were significantly higher than those that had not been exposed to *Nosema* (Figure 2B).

Adult bee survival following *Nosema* exposure, IAPV exposure and co-exposure

For all adult bees (n = 221), there was a significant interaction between IAPV and *Nosema* exposure ($\chi^2 = 4.55$, d.f. = 1, $p = 0.032$). Exposures to both *Nosema* ($p = 0.038$) and IAPV ($p = 0.012$) in isolation decreased survival, relative to unexposed controls (Figure 3). Likewise, bees in the co-exposure treatment had greater mortality ($p = 0.019$), but the survival hazard under co-exposure did not differ from either IAPV ($p = 0.643$) or *Nosema* ($p = 0.643$) single exposures (Figure 3). There was no significant effect of adult body size on mortality ($\chi^2 = 2.16$, d.f. = 1, $p = 0.142$). All analyses were also run with the quantification bees removed (n = 158), but these results were qualitatively identical and had the same statistical patterns.

CHAPTER IV: DISCUSSION

Coinfection of hosts with multiple parasite species is widespread [1], common in nature [9], and range in severity for the impacted host and parasites involved, thus influencing disease ecology and evolution in a variety of ways. This makes studying coinfections and co-exposures a highly relevant challenge for the study of infectious disease, due to a wide range of possible outcomes in dynamic, multi-host, multi-parasite communities [15,97]. Bumble bees in particular are exposed to multiple parasites within the larger plant-pollinator network [40], and are being impacted by emerging infectious diseases that are increasing the novel combinations of parasites they face [66–68]. Studying the effects of sequential exposures of the microsporidian *N. bombi* and the traditionally honey bee virus IAPV, we show the importance of these parasite combinations and that co-exposures can have consequences even if infections do not establish. While exposed, most workers did not become infected with *N. bombi*. Despite this lack of observed infection, we show a significant effect of mortality for bees singly exposed to *Nosema bombi* spores, suggesting that responding to the inoculation did have a cost. We also show that resistance to IAPV is reduced following prior *Nosema* exposure, and workers that were co-exposed to *Nosema* had more intense? IAPV infections compared to their unexposed counterparts (Figure 2). However, increased mortality seen under the IAPV infection alone was not elevated in co-exposed bees. In addition to the results pertaining to effects of prior parasite exposure, to the best of our knowledge, the established experimental infections of IAPV and the survival consequences are also the first documented in a North American bumble bee species.

Increased IAPV infection levels in workers that have been previously exposed to *Nosema* indicate that this prior microsporidian parasite exposure alters resistance to a distinct viral parasite. This outcome is despite the fact that the vast majority of *Nosema* spore exposures

during larval development did not produce viable infections in the adult worker bees. We do not know if infections never established from the *Nosema* exposure or if they were cleared. In either case, the effects of a reduced survival of adults following larval *Nosema* exposure alone and the elevated subsequent IAPV infections indicate that the *Nosema* exposures did interact with the host, changing physiology or condition.

The exact mechanism underlying the decreased resistance to IAPV following the prior *Nosema* exposure could be based on immunity. A costly response to resist a larval *Nosema* exposure could deplete resources to otherwise respond subsequently to the IAPV exposure. Although adult size was not affected, the observed reduced survival of *Nosema* exposed but not infected bees would support a cost of immunity [29]. Alternatively, trade-offs between specific microsporidian and antiviral immune responses could precipitate the outcome. IAPV triggers a specific antiviral RNAi response [98], while the response to a microsporidian *N. ceranae*, related to *N. bombi*, in honey bees has been shown to involve the Toll and IMD immune pathways [99]. As previously noted, immune priming in insects results in enhanced protection from a parasite following a prior exposure infection. However, in this case instead of improving the host's immunity, exposure to a previous infection could decrease the host's immunity to a subsequent distinct infection, and result in enhanced fitness for the secondary parasite. Such "negative" immune priming as a result of a heterologous parasite exposure has not been well studied, but similarly when bumble bee offspring are trans-generationally primed for an antibacterial response they show increased susceptibility to a trypanosome infection [29]. In another instance of increased susceptibility to the second parasite in sequential co-infection interactions, co-infected larvae of *Manduca sexta* that were infected with polydnavirus from a braconid wasp showed increased susceptibility and mortality to *Autographa californica* M

Nucleopolyhedrovirus (AcMNPV) [100]. In this case immunosuppression by the first infection drives the interaction. Such immune suppression is also an intriguing possibility for *N. bombi*. The related *N. ceranae* has been documented to suppress the honey bee host immune response [101]. Further research into the effects of *N. bombi* exposure and infection on bumble bee immunity, including the antiviral response, is warranted to elucidate the underlying mechanism of the pattern of altered viral resistance, but also how exposure to this particular microsporidian may affect other co-infecting parasites of bumble bees.

Whatever the mechanism underlying the change in resistance, these results show that exposure to *Nosema* increases IAPV infection levels, thus potentially increasing transmission potential of IAPV infected workers. This would amplify the IAPV levels present in workers and increase the amount of IAPV within the plant-pollinator network. This amplifies the IAPV levels present in workers and would increase the amount of IAPV within the plant-pollinator network. IAPV is already established as a spillover parasite [67,102,103], indicating that this co-exposure driven increase in transmission potential could exacerbate its effects within the bee community. Increased transmission potential from co-exposure is a very real concern for bumble bees. As *B. impatiens* is a relatively abundant member of the bee community in the eastern United States [49], even if they do not get infected by *N. bombi* at a high prevalence in nature[49], increasing its viral load significantly increases its infectivity and transmission potential. This could ultimately make co-exposed individuals super-spreaders [104] of IAPV or other affected parasites within their communities.

Although the outcome for resistance to IAPV showed the predicted pattern of being reduced on co-exposure, we did not see any discernable effect of *Nosema* prior exposure on survival to IAPV. Both exposures alone significantly increased mortality, but survival data

shows that under combined exposure we see a less than an additive effect, indicating no enhancement of mortality (Figure 3). One potential explanation for the discrepancy between increased infection and no change in survival could be that even the infection levels in the IAPV only exposed bee were already beyond a maximum threshold dictating host mortality. In this case, any subsequent increase in infection levels, as seen here, would not be expected to increase mortality further, even though it would affect transmission, as mentioned above. However, if similar effects were seen at lower infection levels, which could result from lower initial exposures to IAPV, then we would expect that the boost in virus numbers would have corresponding consequences for bee survival.

As already raised, it is important to note that our infection quantification and microscopy results indicate that our bees either had extremely low levels of *N. bombi* present, below the level of detection, or no *Nosema* at all as adults. This is inconsistent with prior work using the same methods that established robust *N. bombi* infections in *Bombus impatiens* males [76]. The infection outcome differences could be due to different susceptibility of males versus the workers used, and the haploid-susceptibility hypothesis posits that haploid male insects have increased likelihood of severe infection to disease relative to diploids [105]. Despite this, Ruiz-González and Brown [106] did not see differences between workers and males of the European bumble bee *Bombus terrestris* when infected with the gut trypanosome *Crithidia bombi*. However, instances of increased susceptibility to *N. ceranae* in honey bee drones has been observed [107]. Another potential explanation is that due to logistical constraints, in this study we used a different isolate of *N. bombi* than the previous study [76]. Although isolated in the same way, this isolate came from *B. terricola* sourced in Vermont, USA, whereas the isolate previously used came from *B. occidentalis* collected in Oregon, USA. *Nosema bombi* has been

suggested to lack genetic diversity in the USA [48], but isolate specific infection outcomes under similar conditions could suggest otherwise.

Understanding how infection outcomes for hosts and parasites are influenced by co-exposures and coinfections, which will be frequent in nature, is important in the fields of disease ecology and ecological immunology [15], which seek to explain natural variation in infection outcomes. Furthermore, from the perspective of bumble bee conservation, understanding how multiple parasites interact to affect bumble bees is critical to understanding factors that threaten the population health of these important pollinators. We demonstrate that even non-establishing larval microsporidian parasite exposures can negatively affect adult worker health, through reduced survival. Furthermore, this prior exposure negatively affects adult resistance to a subsequent viral infection, which will likely have consequences for host individual and colony health and viral transmission dynamics. Especially as parasite dynamics are shifting in response to global changes [108] and as bees become exposed to novel combinations of parasites [17], studies like this are imperative to show how interactions between multiple parasites within individual hosts can alter host-parasite outcomes and dynamics.

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APPENDIX A: FIGURES

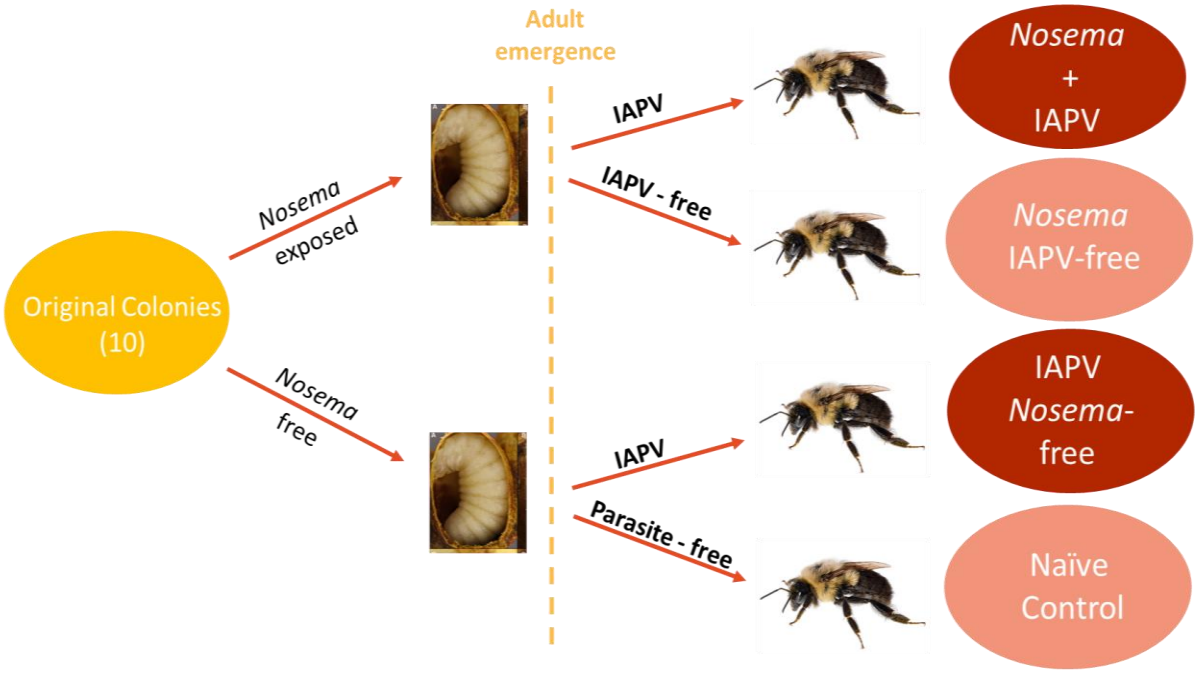

Figure 1. Overview of experimental co-exposure set-up. Larval brood clumps from eight original colonies were split into microcolonies, either exposed to *N. bombi* or left naïve. Upon adult emergence (dashed orange line), bees were isolated individually and either exposed with IAPV or not, resulting in four possible treatment combinations: co-exposed, *Nosema* exposed, IAPV exposed, or unexposed.

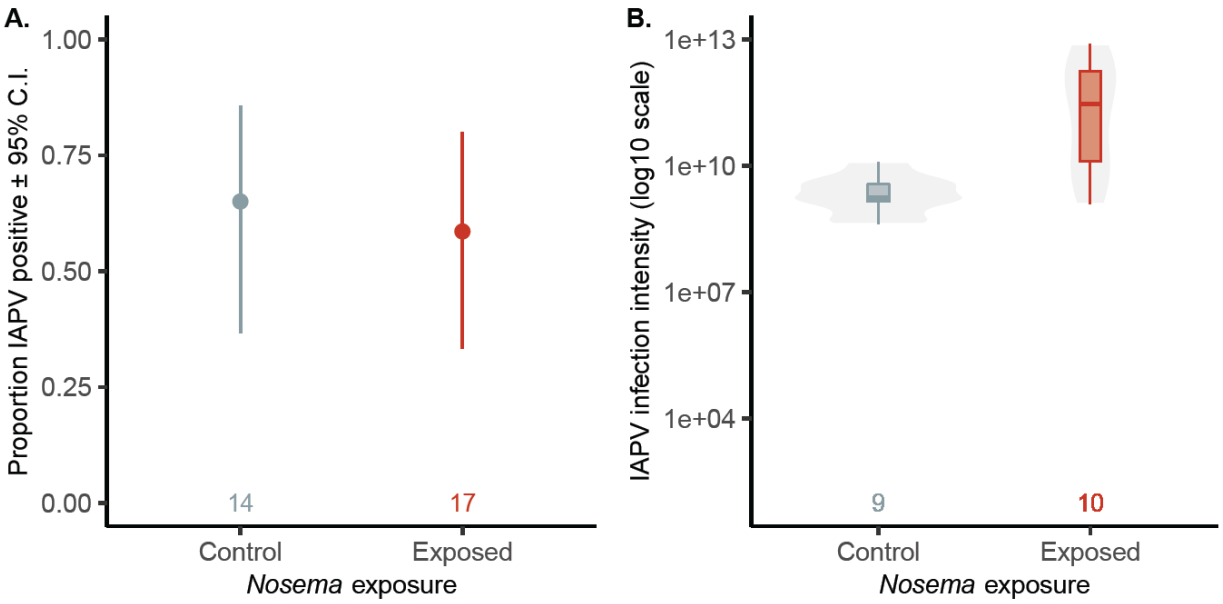

Figure 2. The influence of prior *Nosema* exposure on IAPV infection. A) Proportion of IAPV positive individuals (estimated marginal means with 95% confidence intervals). B) Violin plots with nested boxplots showing IAPV infection intensities in IAPV positive individuals. Dark horizontal lines within 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. The number of samples is noted along the X axis.

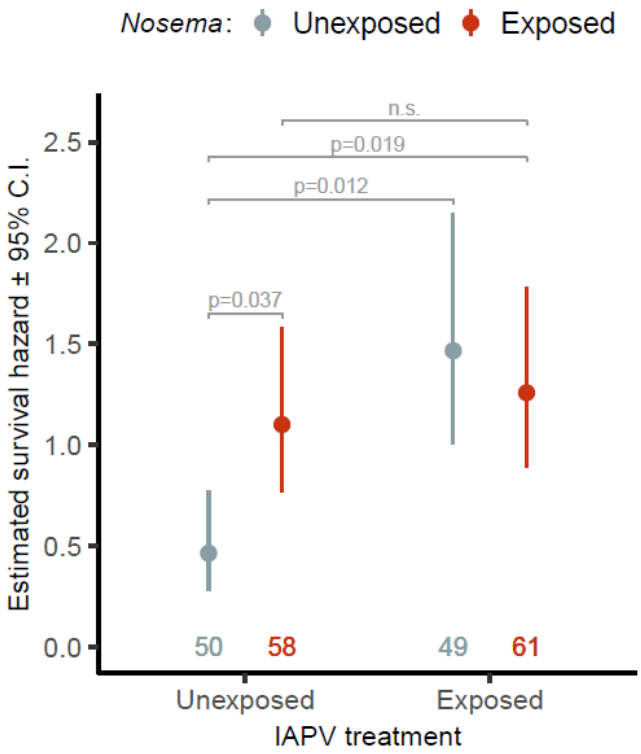

Figure 3. Estimated survival hazard in adult worker bees depending on IAPV and *Nosema* exposure treatments. Points represent the estimated marginal means, and bars represent 95% confidence intervals. Sample size is noted along the X axis.