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ORIGINAL RESEARCH

Consequences of microsporidian prior exposure for virus infection outcomes and bumble bee host health

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Abstract

Host–parasite interactions do not occur in a vacuum, but in connected multi-parasite networks that can result in co-exposures and coinfections of individual hosts. These can affect host health and 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. *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 double parasite exposure will also reduce host tolerance of infection, as measured by host survival. Although our larval *Nosema* exposure mostly did not result in viable infections, it partially reduced resistance to adult IAPV infection. *Nosema* exposure also negatively affected survival, potentially due to a cost of immunity in resisting the exposure. There was a significant negative effect of IAPV exposure on survivorship, but prior *Nosema* exposure did not alter this survival outcome, suggesting increased tolerance given the higher IAPV infections in the bees previously exposed to *Nosema*. These results again demonstrate that infection outcomes can be non-independent when multiple parasites are present, even when exposure to one parasite does not result in a substantial infection.

Keywords Host-parasite interactions · Coinfection · Co-exposure · Bumble bee · *Nosema bombi* · IAPV

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 (Rigaud et al. 2010). 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. Such model pairwise systems have provided significant advances in our knowledge of host–parasite interactions (e.g., Altizer et al. 2004; de Roode et al. 2008; Sadd and Barribeau 2013), but 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 (e.g., López-Villavicencio et al. 2007; West et al. 2015; Mwangi et al. 2006; Hartgers and Yazdanbakhsh 2006). The interdependence of infection outcomes during coinfection can lead to changes in parasite transmission and virulence relative to single infections (Rigaud et al. 2010; Alizon et al. 2013). Thus, coinfections are likely to have wide-reaching consequences for the health of hosts, parasite disease ecology, and host–parasite evolution (Alizon 2013a, b; Susi et al. 2015; Seppälä and Jokela 2016). Although infection outcomes may not be modified in all cases of co- versus single infections

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(Seppälä et al. 2009), variation in coinfection dynamics makes studying co-exposures and coinfections resulting from multi-parasite networks a central open topic in disease ecology (Lively et al. 2014). This is particularly relevant given ongoing global change that has the potential to bring hosts into contact with novel combinations of parasites and other stressors (Meeus et al. 2011; Telfer and Bown 2012).

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 (Holt and Dobson 2006; Pedersen and Fenton 2007; Graham 2008). In the water flea *Daphnia magna*, bacterial and microsporidian coinfection had detrimental consequences for the host and both parasites (Ben-Ami et al. 2011), 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 (Sandland et al. 2007). Whether such co-exposures are simultaneous or sequential and the order of sequential exposures can also determine infection outcomes (Karvonen et al. 2019). 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 (Hoverman et al. 2013).

The underlying mechanisms that determine differential outcomes under a scenario of co-exposure or coinfection will vary between the hosts and 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 (Graham 2008). 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 (Moret and Schmid-Hempel 2000; Sadd and Schmid-Hempel 2009a). For example, internal trade-offs between specific arms of the immune system (Pedersen and Fenton 2007; Sadd and Schmid-Hempel 2009b) may lead to sub-optimal responses by the host when responding to multiple parasites. Additionally, immune suppression by one parasite can also facilitate another (Ezenwa et al. 2010). 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. The balance of the costs of immunity, 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 relevance for areas such as conservation or pest control, we still lack an understanding of the effects of coinfection on infection and host-parasite dynamics. Bumble bees (*Bombus* spp.), wild and managed pollinators of ecological, economic, and conservation concern are exposed to multiple parasites in a multi-species network (Cameron and Sadd 2020). A key bumble bee parasite, also touted to be associated with the declines of some bumble bee species, is the microsporidian *Nosema (Vairimorpha) bombi* (Cameron et al. 2011; Cameron et al. 2016). 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 (McIvor and Malone 1995; Franzen 2004; Franzen et al. 2005). The parasite is subsequently found in multiple tissues (Fries et al. 2001), causing considerable tissue damage for individual bees. Additionally, as a microsporidian, *N. bombi* manipulates the host's mitochondrial machinery to hijack energy (Tsaousis et al. 2008; Keeling 2009), which will drain host energy stores and could cause physiological problems (Masson et al. 2017). *Nosema bombi* infection has been shown to reduce queen colony founding success (Van Der Steen 2008), male and worker longevity in the laboratory (Otti and Schmid-Hempel 2007), and the size of field colonies (Otti and Schmid-Hempel 2008). Its high potential virulence and the fact that its prevalence of infection is highest in declining North American bumble bee species (Cameron et al. 2011; Cameron et al. 2016) make studying the context dependence of infection outcomes highly relevant. Despite *N. bombi* being nested in a community of bumble bee parasites, we currently have limited information on coinfection outcomes.

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 (Colla et al. 2006; McMahon et al. 2015; Graystock et al. 2016b). 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 (Singh et al. 2010; Fürst et al. 2014; Dolezal et al. 2016; Graystock et al. 2016b; Wilfert et al. 2016; Alger et al. 2019; Manley et al. 2019; McNeil et al. 2020). Spillover may create problems for the native bumble bee community, altering the risk and identity of infections (Piot et al. 2022). For example, Israeli Acute Paralysis Virus (IAPV) has been found at high levels in bumble bees close to infected honey bee apiaries (Singh et al. 2010). 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 (Boncristiani et al. 2013). IAPV invades almost all honey bee tissues, causing decreased motor function, severe muscle spasms, paralysis, and death (Chen et al. 2014; Galbraith et al. 2015). 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 (Wang et al. 2016; Wang et al. 2018). 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, altering 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) elevated both lethal and sub-lethal effects of infection (Graystock et al. 2016a). Our work presented here investigates how sequential exposures of *N. bombi* in larvae and IAPV in adults influence infection outcomes in the bumble bee *Bombus impatiens*. The overarching hypothesis is that infection outcomes of *N. bombi* and IAPV will be modified by sequential co-exposures and coinfection, with consequences for host-parasite dynamics, including host infection resistance and tolerance. Resistance here is defined as the ability to prevent and limit the infectious burden, while tolerance is the ability to mitigate the health impact associated with a given parasite infection intensity (Schneider and Ayres 2008; Baucom and de Roode 2011). Microsporidian infection increases host cell energy metabolism during infection (Dolgikh et al. 2002; Hu et al. 2021) and a related bee microsporidian, *N. ceranae*, has been implicated in suppressing immune function (Chaimanee et al. 2012; Paris et al. 2018; Macías-Macías et al. 2020). 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, because of such effects. Subsequently, co-exposure and coinfection of the two individually determinantal infections are predicted to further reduce host survival.

Materials and methods

Overall experimental design

Experimental coinfections were performed to evaluate how time lagged exposures of *N. bombi* and IAPV in *B. impatiens* influence 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 the following four exposure treatments: (1) co-exposed to *Nosema bombi* and IAPV, (2) *Nosema* exposed, (3) IAPV exposed, or (4) unexposed (Fig. 1). Subsequently, infection

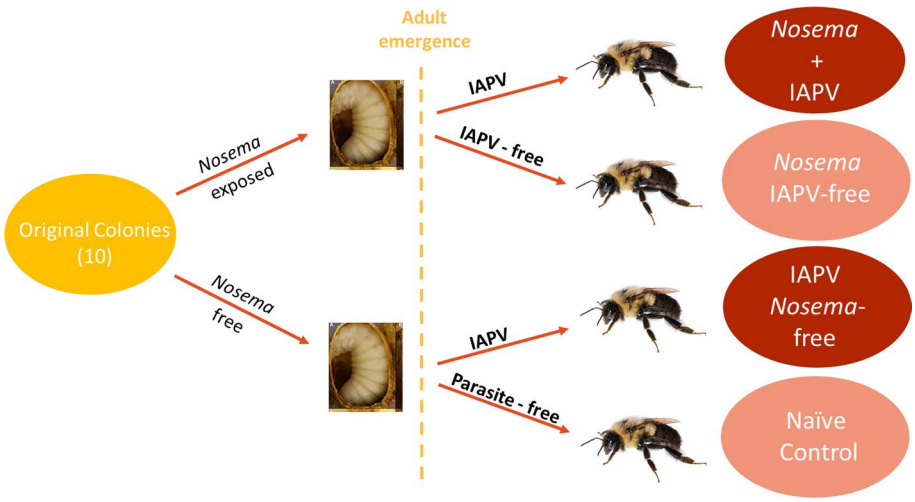

Fig. 1 Overview of experimental co-exposure set-up. Larval brood clumps from eight original colonies were split into microcolonies, either exposed to *N. bombi* or left naïve. Upon adult emergence (dashed orange line), bees were isolated individually and either exposed with IAPV or not, resulting in the following four possible treatment combinations: co-exposed, *Nosema* exposed, IAPV exposed, or unexposed. A total of 218 bees were assayed, with 61 being assessed for fixed timepoint infections and the remainder maintained for survival responses

outcomes of host infection resistance (infection loads) and survival were assessed. A total of 61 adult workers were assessed for set time point infections and 157 adult workers were maintained for survival. For the infection quantification, these bees came from 13 *Nosema* exposed and 12 unexposed microcolonies set up from eight original source colonies. For survival, these bees came from 16 *Nosema* exposed and 14 unexposed microcolonies set up from seven original source colonies.

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 (400× 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 Calhoun et al. (2021), quantified, and were stored at − 80 °C until use in experimental inoculation.

The IAPV inoculum used to expose the bees was identical to the one used and described in Hsieh et al. (2020a), produced per methods of Hsieh et al. (2020b). In short, an IAPV inoculum was produced via injection into honey bee pupae, then purified and extracted, resulting in an inoculum containing IAPV particles at 99.79% purity with 5.45×10^{10} IAPV particles/µl. 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 these preliminary trials, the stock inoculum was diluted to 5×10^{-9}, bringing it to an estimated concentration of approximately 273 IAPV particles/µl. Each bee was then injected with 2 µl of this inoculum, thus receiving a treatment of an estimated 546 IAPV particles. This is similar to the work of Wang et al. (2018), which infected another bumble bee species, *B. terrestris*, with IAPV using an exposure of 500 particles/bee. In our preliminary data, this dose resulted in 75% bee mortality between 5 and 10 days after inoculation. The prepared solution was stored at − 80 °C until use in experimental inoculations.

Microcolony preparation and *Nosema* inoculation

Original source colonies were monitored until second-instar larval brood were present in the colony. This brood of, on average, seven larvae was 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 (Klinger et al. 2019). 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 h of microcolony acclimation, each larva within *Nosema*-exposed microcolonies was individually inoculated with 40,000 *N. bombi* spores in 2 µl of a sugar water and pollen solution (Calhoun et al. 2021). 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 × 10 × 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 min. Once anesthetized, bees were injected between the first and second abdominal tergite with 2 µl of either their pre-assigned viral inoculum (546 IAPV particles per bee) 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 (Graystock et al. 2015), injections provide practical benefits, such as improved visibility and robustness of infection for controlled experimental inoculations (Wang et al. 2018). 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 (Core et al. 2012; Menail et al. 2016; Wang et al. 2018). In addition, parasitic mites of honey bees, such as *Varroa destructor*, act as a virus vector (Martin et al. 2012; Ryabov

et al. 2014) and transmit IAPV. 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* (Goka et al. 2006; Yoneda et al. 2008), carry such a potential.

Infection outcomes: survival and infection prevalence and intensities

Worker survival was monitored daily ($n = 157$). A random subset of bees ($n = 61$) was killed 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 (Cartar 1992). 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 (Müller and Schmid-Hempel 1992; Müller et al. 1996), 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. (2021). 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/µl), and pJET1.2 Cloning Vector (1 ng/µl), with three 2.4 mm 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 µl for RNA extraction, 250 µl volume for *Nosema* DNA extraction, and the remainder for microscopy. For RNA extraction, 750 µl of TRIzol LS was added and samples placed at − 80 °C until further extraction. The homogenate for DNA extraction and microscopy were placed at − 20 °C.

To extract IAPV RNA from the samples, each homogenate with TRIzol LS was removed from − 80 °C and incubated at room temperature for 15 min. 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 s. Samples were then incubated at room temperature for 5 min, before centrifugation at 4 °C for 10 min 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 min and then centrifuged for 10 min 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 min 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 min. 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 and concentrations were checked 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′) (Carrillo-Tripp et al. 2016; Geffre et al. 2020). Reverse transcription took place at 50 °C for 25 min, then an initial denaturation step of 95C for 5 min, 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 s and 55 °C for 30 s with stepwise increases of 0.5 °C from 55 to 95 °C, as detailed in Carrillo-Tripp et al. (2016). RNA250 quantification used primers from de Miranda et al. (2021) with initial denaturation of 10 min at 95 °C, followed by 40 amplification cycles of 15 s denaturation at 95 °C and a simultaneous annealing and extension at 58 °C. To account for between PCR variability, each qPCR plate contained a synthetic standard serial dilution curve of an Integrated DNA Technologies™ gBlock of the target product sequence, which was also used to verify primer amplification efficiency (> 95%).

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 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′- GGCCCA TGCATGTTTTTGAAGATTATTAT-3′, reverse: 5′- CTA CACTTTAACGTAGTTATCTGCGG-3′) (Plischuk et al. 2009). Initial denaturation took place for 10 min at 95 °C, followed by 40 amplification cycles of 15 s denaturation at 95 °C and a simultaneous annealing and extension at 58 °C (Chu and Cameron 2017). An additional qPCR plate quantifying the passive nucleic acid reference standard pJET1.2 was also performed (de Miranda et al. 2021). Each qPCR plate again contained a synthetic standard serial dilution curve of an Integrated DNA Technologies™ gBlock of the

target product sequence to account for between PCR variability and verify primer amplification efficiency.

Each qPCR 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 bee sample that were corrected for any differential extraction efficiencies between samples.

The remaining homogenate was used for phase contrast microscopy at 400× 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 (Bates et al. 2007) and *glmmTMB* package (Brooks et al. 2017), respectively. For survival, Mixed Effects Cox Proportional Hazards models were fit using the package *coxme* (Therneau et al. 2015). 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 (Lenth et al. 2020). 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.

Results

Nosema exposure during development and adult size

There was no significant effect of larval exposure to *Nosema* on adult size ($F = 0.929$, $df = 1$, 22.43, $p = 0.345$). Mean radial cell lengths, a surrogate for body size, were 2.55 mm (s.e. = 0.025 mm, $n = 99$) for unexposed bees and 2.48 mm (s.e. = 0.031 mm, $n = 119$) 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 total infection level of 1,850,672 *Nosema* genome copies per bee. In addition, all samples were screened microscopically for the presence of *Nosema* extracellular spores, with spores only detected in the aforementioned sample. Further analyses were performed with this positive individual present and removed, and we observed no quantitative difference. 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 ($\chi^2 = 0.140$, $df = 1$, $p = 0.708$, Fig. 2A) nor body size ($\chi^2 = 0.576$, $df = 1$, $p = 0.448$) significantly influenced if bees showed IAPV positive infections. Body size also did not significantly affect IAPV infection intensities ($\chi^2 = 0.300$, $df = 1$, $p = 0.585$). However, prior exposure of bees to *Nosema* had a significant effect on IAPV levels in infected bees ($\chi^2 = 41.27$, $df = 1$, $p < 0.0001$). 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* (Fig. 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 ($\chi^2 = 4.55$, $df = 1$, $p = 0.032$). Exposures to both *Nosema* ($p = 0.038$) and IAPV ($p = 0.012$) in isolation decreased survival, relative to unexposed controls (Fig. 3, Supplementary Figure S1). Likewise, bees in the co-exposure treatment had greater mortality than unexposed bees ($p = 0.019$), but the survival hazard under

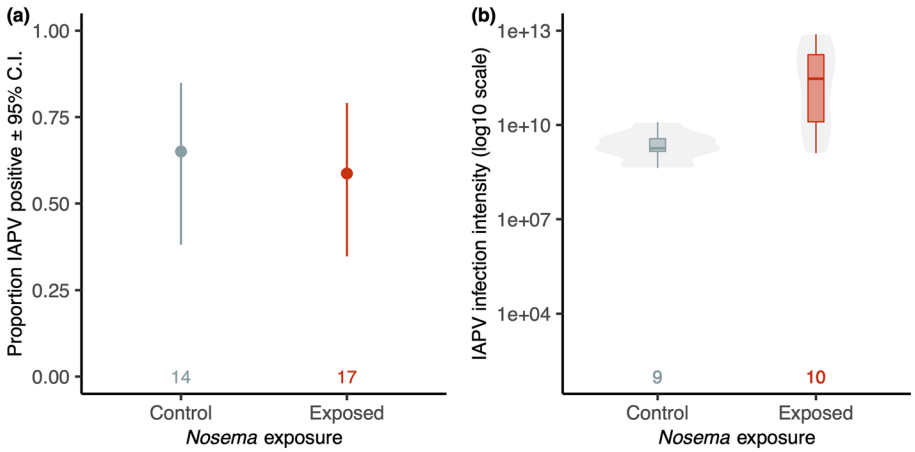

Fig. 2 The influence of prior *Nosema* exposure on IAPV infection. **a** Proportion of IAPV positive individuals (estimated marginal means with 95% confidence intervals). **b** Violin plots with nested boxplots showing IAPV infection intensities in IAPV positive individuals differing between bees previously exposed or not to *Nosema* ($\chi^2 = 41.27$, $df = 1$, $p < 0.0001$). Dark horizontal lines within each box indicate 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

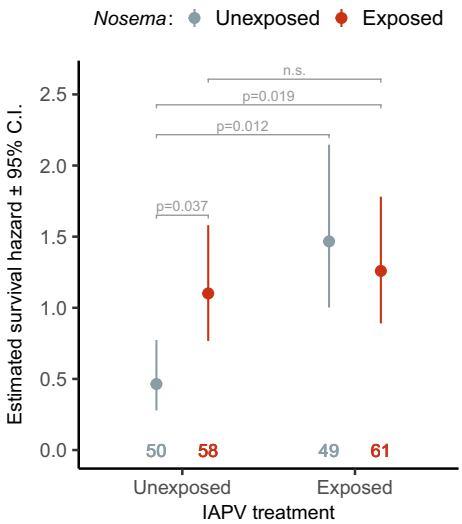

Fig. 3 Estimated survival hazard in adult worker bees depending on IAPV and *Nosema* exposure treatments. Higher survival hazards represent reduced survival, and points represent the estimated marginal means and bars 95% confidence intervals. Sample size is noted along the *X* axis. *p* values from FDR corrected pairwise tests of significance between groups is shown above the bars

co-exposure did not differ from either IAPV ($p = 0.643$) or *Nosema* ($p = 0.643$) single exposures (Fig. 3). There was no significant effect of adult body size on mortality ($\chi^2 = 2.16$, $df = 1$, $p = 0.142$). All survival analyses were also run with the quantification bees removed ($n = 158$), as their inclusion as censored values could potentially change the outcome of the analyses, but these results were qualitatively identical and had the same statistical patterns.

Discussion

Coinfection of hosts with multiple parasite species is widespread (Rigaud et al. 2010), common in nature (Alizon et al. 2013), 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 (Alizon and Van Baalen 2008; Lively et al. 2014). Bumble bees in particular are exposed to multiple parasites within the larger pollinator network (Cameron and Sadd 2020) and are being impacted by emerging infectious diseases that are increasing the novel combinations of parasites they face (Singh et al. 2010; Dolezal et al. 2016; Piot et al. 2022). Studying the effects of sequential exposures of the microsporidian *N. bombi* and IAPV, a virus typically associated with honey bees, we show that co-exposures can have consequences even if infections do not establish. While exposed, all but one worker did not become infected with *N. bombi*. Despite this lack of ongoing infection, we show a significant effect on 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 partially reduced following prior *Nosema* exposure, and workers that were co-exposed to *Nosema* had more intense IAPV infections compared to their unexposed counterparts (Fig. 2). However, increased mortality seen under the IAPV infection alone was not further elevated in co-exposed bees.

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 even though the vast majority of *Nosema* spore exposures during larval development did not produce viable infections in the adult worker bees. Although non-sporulating infections have been detected in nature (Blaker et al. 2014), our molecular screen shows this is not the case in this study, demonstrating that absence of spores reflected absence of infection in adults. We do not know if infections never established from the *Nosema* exposures or if infections established but they were cleared. In either case, the outcomes of a reduced survival of adults following larval *Nosema* exposure alone and the elevated IAPV infections following earlier *Nosema* exposure indicate that these *Nosema* exposures did indeed 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 (Sadd and Schmid-Hempel 2009). Alternatively, trade-offs between specific microsporidian and antiviral immune responses could precipitate the outcome. IAPV triggers a specific antiviral RNAi response (Cappelle et al. 2016), 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 (Li et al. 2017). In another instance of increased susceptibility to the second parasite in sequential co-infection interactions, co-infected larvae of *Manduca sexta* infected with polydnavirus from a braconid wasp showed increased susceptibility to *Autographa californica* M Nucleopolyhedrovirus (AcMNPV) (Washburn et al. 2000). In this case immunosuppression by the first infection drives the interaction, and such immune suppression could be a possibility for *N. bombi* given the related *N. ceranae* has been documented to suppress the honey bee host immune response (Antúnez et al. 2009). Further research into the effects of *N. bombi* exposure and infection on bumble bee immunity, including the antiviral response, is warranted not only 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.

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 (Calhoun et al. 2021). 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 (O'Donnell and Beshers 2004). Despite this, Ruiz-González and Brown (2006) 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 (Retschnig et al. 2014). Another potential explanation is that due to logistical constraints, this study used a different isolate of *N. bombi* than the previous study (Calhoun et al. 2021). Although the *Nosema* used here was isolated by the same protocol, it 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 between isolates in the USA (Cameron et al. 2016), but isolate specific infection outcomes under similar conditions could suggest otherwise.

Although the outcome for resistance to IAPV showed the predicted pattern of being partially 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 show that under combined exposure we see a less than an additive effect, indicating no enhancement of mortality (Fig. 3). Interestingly, the pattern of increased levels of infection of IAPV following prior *Nosema* exposure, but no concurrent further decrease in survival, is indicative of higher tolerance of infection. This outcome is counter to our predictions, but it could have important consequences for transmission. Higher infection tolerance is suggested to increase parasite transmission (Baucom and de Roode 2011; Adelman and Hawley 2017). The consequence of exposure to *Nosema* increasing IAPV infection levels without further reducing survival could be increased IAPV transmission potential. This would amplify the IAPV levels present in workers and increase the amount of IAPV within the pollinator network. IAPV is already established as a spillover parasite (Levitt et al. 2013; Singh et al. 2010; Dalmon et al. 2021), indicating that such a 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 (Cameron et al. 2011), even if they do not get infected by *N. bombi* at a high prevalence in nature (Cameron et al. 2011), increasing its viral load significantly increases its infectivity and transmission potential. This could ultimately make co-exposed individuals super-spreaders (Streicker et al. 2013) of IAPV or other affected parasites within their communities.

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 (Lively et al. 2014), 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 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 (Jolles et al. 2008) and as bees become exposed to novel combinations of parasites (Meeus et al. 2011), 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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Author contribution statement ECM, AGD, and BMS conceived and designed the experiments. ECM performed the experiments. ORC performed microscopy and assisted with data collection. ECM and BMS analyzed the data. ECM and BMS wrote the manuscript; AGD provided editorial advice.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

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