Testing the multiple stressor hypothesis: Chlorothalonil exposure alters transmission potential of a bumblebee pathogen but not individual host health

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ABSTRACT

Numerous threats are putting pollinator health and essential ecosystem pollination services in jeopardy. Although individual threats are widely studied, their co-occurrence may exacerbate negative effects, as posited by the multiple stressor hypothesis. A prominent branch of this hypothesis concerns pesticide-pathogen co-exposure. A landscape analysis demonstrated a positive association between local chlorothalonil fungicide use and microsporidian pathogen (*Nosema bombi*) prevalence in declining bumble bee species (*Bombus spp*.), suggesting an interaction deserving further investigation. We tested the multiple stressor hypothesis with field-realistic chlorothalonil and *N. bombi* exposures in worker-produced *B. impatiens* microcolonies. Chlorothalonil was not avoided in preference assays, setting the stage for pesticide-pathogen co-exposure. However, contrary to the multiple stressor hypothesis, co-exposure did not affect survival. Bees showed surprising tolerance to *Nosema* infection, which was also unaffected by chlorothalonil exposure. However, previously fungicide-exposed infected bees carried more transmission-ready spores. Our use of a non-declining bumblebee and potential higher chlorothalonil exposures under some scenarios, could mean stronger individual or interactive effects in certain field settings. Yet, our results alone suggest consequences of pesticide co-exposure for pathogen dynamics in host communities. This underlies the importance of considering both within- and between-host processes when addressing the multiple stressor hypothesis in relation to pathogens.

INTRODUCTION

The ecological and economic contributions of wild native bee communities [1] make conserving their diversity and understanding the threats they face of paramount importance. The abundance and distribution of populations of several native bumblebee species have been significantly reduced over recent decades, including in Europe [2], the Americas [3,4], and Eastern Asia [5]. In North America, population reductions have been noted by global and federal agencies, and, based on International Union for Conservation of Nature (IUCN) listings, 26% of evaluated North American species are threatened [6].

Declines of bumblebees and other insect pollinators may be precipitated by a suite of environmental stressors that threaten population health and thus essential ecosystem services [6,7]. Suggested major stressors include climate change [8], habitat degradation and fragmentation [9,10], pesticides [11–15], and pathogen infection [7,16,17]. Undoubtedly, such factors will not only act in isolation, but stressor combinations may present a greater threat to bee health if effects of co-exposure are additive or worse [6,7].

Recently, the combined effects of pathogen infection and pesticide exposure have gained attention in relation to bumblebee declines [12,14,18]. Co-exposure of pathogens and pesticides may exacerbate the individual negative effects of each stressor alone, for example through interactions between pesticides and immunity [19–21]. Negative effects may also be amplified by co-exposure if each stressor contributes independently or synergistically to a reduction in individual or colony condition. The concept that stressors, such as pesticides and pathogens, interact to amplify the detrimental effects to a host has been termed the “multiple stressor hypothesis” [6,22]. Thus far, tests of this hypothesis in bumblebees have been carried out predominantly in a single host species and have been largely focused on model pathogen systems [e.g., 12,14]. Relevant threats potentially associated with population declines should be empirically tested under this framework to aid in our understanding of declines and inform remediation.

In North America, a microsporidian pathogen of bumblebees, *Nosema bombi*, has been linked with declines over recent decades, with a higher infection prevalence in declining species relative to stable species [16,23]. Evidence suggests that increased prevalence in declining species have occurred recently [23]. Studies on the European *B. terrestris* demonstrate severe reductions in individual and colony level traits associated with fitness upon infection [24–26]. Infected bees shed transmission-ready extracellular spores, which eject a polar filament into epithelial cells to initiate infection in a new suitable host, followed by further within-host replication of these activated intracellular stages [27,28]. Due to its documented virulence, *N. bombi* is considered to be an important emerging or re-emerging infectious disease in bumblebees [29]. The touted association between this microsporidian and bumblebee declines make it an important pathogen with which to test the multiple stressor hypothesis.

Bumblebee immunity will be critical to resist and tolerate pathogen infection, as demonstrated for other *Nosema spp.* in honeybees [30,31]. Thus, any co-occurring stressors that weaken immunity, such as sublethal exposure to pesticides [19,20], should be incorporated empirically into the multiple stressor framework. Much recent work has focused on systemic neonicotinoids [e.g., 12,20,21], however, a largely overlooked pesticide in experimental studies, but one that may influence bumblebee health, especially in relation to pathogen interactions, is the non-systemic fungicide chlorothalonil. This fungicide, intended to inhibit enzymatic processes involved with cellular respiration of fungal cells [32,33], has large-scale agricultural application, with many target crops being bumblebee pollinated [34]. It is currently not approved for use in the European Union, but no such restrictions exist in the United States of America [35]. Interestingly, a follow up to the demonstration of higher *N. bombi* prevalence in declining North American bumblebee species [16] linked chlorothalonil to natural pathogen prevalence [36]. Landscape use of chlorothalonil was the strongest predictor of *N. bombi* prevalence in declining bumblebee species [36]. This study is suggestive of an interaction, but there has been no experimental verification that would support a causative link between chlorothalonil and *N. bombi* infection, and the consequences of co-exposure for bumblebee health.

The fungicide chlorothalonil and the microsporidian *N. bombi* are stressors that may have the potential to act together to the detriment of bumblebee health. A preference of honeybees for chlorothalonil-laced sugar water [37] indicates that bees may not avoid chlorothalonil-contaminated resources, and hence any effects of its exposure. Few studies have investigated chlorothalonil's influence on bee health, but interference of larval and pupal development, and altered nutrition and social immunity are demonstrated negative consequences of sublethal exposure [38–40]. Furthermore, chlorothalonil may interact with invertebrate immunity, with studies in bivalves showing effects on cellular immunity [41]. Thus, chlorothalonil exposure may have direct or indirect interactions with immunity, with subsequent consequences for host defense against infection. However, experiments incorporating the combined stressors of chlorothalonil and *N. bombi* are needed in order to offer a mechanistic explanation for the correlative relationships seen in nature [36] and bumblebee declines [16].

In line with the multiple stressor hypothesis, we hypothesize that, due to direct or indirect interactions with bumblebee immunity, exposure to sub-lethal doses of the fungicide chlorothalonil will reduce resistance and tolerance to *N. bombi* infection, thus exacerbating detrimental effects on bee health. We predict that: (1) like honeybees [37], bumblebee workers will not avoid field realistic chlorothalonil doses; (2) exposure to chlorothalonil alone will result in decreased survival and condition, as measured by survival and a protein biochemical assay; (3) *Nosema bombi* infection will have previously described detrimental effects; and (4) concurrent *Nosema* and chlorothalonil exposure will result in increased susceptibility to infection, higher infection loads, and reduced tolerance to infection. We use worker-produced microcolonies of the bumblebee *B. impatiens*, with the caveat that this is a non-declining species [16], but it is used in this initial study due to feasibility of working with declining species in the laboratory at this scale.

METHODS

Overall study design

Preference assays were carried out to assess if individual adult worker bumblebees differ in consumption of chlorothalonil-spiked or control sugar water when given one or the other (no choice assay) or presented with both (choice assay). To assess individual and combined effects of chlorothalonil and *N. bombi*, worker produced microcolonies established from eight source colonies were used to administer four treatment combinations: 1) chlorothalonil exposure (n=19 microcolonies); 2) *N. bombi* exposure (n=20); 3) chlorothalonil and *N. bombi* co-exposure (n=24); 4) control (n=19) (Figure S1). After separation from the queen, a dominant worker develops ovaries, laying haploid eggs that develop as males [42]. Such microcolonies allow for exposure treatments at a specific larval stage, greater replication of a simulated colony setting, and administration of treatment combinations across the same colony genetic background. Microcolony development and production was recorded, and produced males were assessed for body size, protein amounts, total infection intensity, extracellular spore loads, and survival (Figure S1).

Bumblebee source colonies

For the preference assays, individual *B. impatiens* workers were acquired from five lab-reared colonies from field-caught queens, and one commercial colony (Koppert Biological Systems, Howell, Michigan, USA). For the microcolony experiment, eight commercial source colonies were used. 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.). All established colonies were confirmed free of common pathogen infections and maintained under standard laboratory conditions (S1 Text).

Chlorothalonil treatments

Chlorothalonil was provided at 100 ppb in sugar water and pollen. Due to the feasibility of replicating the individual and co-exposure treatment regimes, we were constrained to a single concentration. The chosen concentration was deemed a reasonable approximation to levels found in plant nectar (76ppb) and pollen (265ppb) [35], but it is important to note that while an average around 100 ppb has been detected in honeybees [43], considerably higher concentrations of residues have been found in honeybee collected pollen [40,44]. Based on estimated daily consumption [44], larvae in our exposure regime would be expected to consume 8ng of chlorothalonil, which again is a reasonable approximation of estimated average dietary exposure of bumblebee larvae to chlorothalonil (5.84ng), but considerably below the estimated possible maximum dietary exposure (544ng) [44]. Chlorothalonil (Millipore Sigma, 36791) stock solutions were prepared, and dilution to 100 ppb chlorothalonil was performed immediately prior to use. Dimethyl sulfoxide (DMSO) was used in stock preparation, and therefore a comparable amount was used in control exposures (S2 Text).

Nosema bombi preparation

A *N. bombi* isolate (lab unique ID O17.01) was sourced from an infected *B. occidentalis* colony, and preliminary studies had verified infection in *B. impatiens* larvae. After a standard extraction protocol, aliquots of spore solutions were stored at -80°C until experimental inoculation, when they were suspended in a sugar water and pollen solution at 10,000 spores/µL (S3 Text).

Chlorothalonil preference assays

Isolated individuals in the no-choice assay were provided with a single feeder with 100 ppb chlorothalonil-spiked or control sugar water. Workers were isolated into plastic containers (10 x 5 x 8 cm) with an upturned 0.65 mL microcentrifuge tube feeder, modified with two 1.6 mm diameter feeding holes in the base. For the choice assay, a separate set of individuals were isolated into similar containers, but with two feeders providing both chlorothalonil-spiked or control sugar water to each bee. Consumption was measured over two time periods, zero to two- and three to six-days post-initiation, with feeders replaced between periods. The change in feeder mass to the nearest milligram (XA Analytical Balance, Fisher Scientific) relative to the mean of 10 pre-weighed reference standards determined sugar water consumption [45].

Microcolony set-up: chlorothalonil and *Nosema* exposures

Microcolonies were generated by isolating four randomly chosen workers from the queenright colony into a holding container (16.5 x 11.5 x 11.5 cm). Upon a clay-based substrate (Tidy Cats), three 60 mm diameter petri dishes held either worker-established brood laid upon a starting pollen pellet (2:1 blend of sugar water and ground pollen), a 15 mL sugar water feeder, or a second pollen pellet for subsequent treatments/feeding. All microcolonies were housed under red-light illumination at 26 +/- 1.5°C.

At six-days after the first oviposition in each microcolony, when larvae would be in their first to second instar [46], microcolonies received 5 mL of sugar water and 1 g pollen pellets with either 100 ppb chlorothalonil or control. Subsequently, at eight-days post-oviposition, individual larvae received a 2 μL inoculum of either 20,000 *N. bombi* spores suspended in a sugar water/pollen solution or a comparable control solution without spores (S3 Text). Additionally, treatment sugar water and pollen pellets were replaced at this time, and again at 10-days post-oviposition. At 12-days post-oviposition, chlorothalonil treatments ceased, and all subsequent provisions for the remainder of development were untreated. Sugar water consumption was measured as volume change over each timepoint. Pollen pellet remnants were dried at 55°C for two days, then consumption was recorded as the mass change relative to 10 pre-weighed standards. Microcolonies were monitored daily until adult eclosion.

Development time and adult size

The number of individual larvae receiving inocula was recorded and compared to the number that eclosed as adults, allowing us to infer how treatments affected progression through development. Emerging individuals were removed within 24 hours, and isolated into plastic containers with sugar water provided *ad libitum*. Based on observations of microcolony development, isolating males from microcolonies concluded at 37 days post-oviposition to ensure that individuals collected had been exposed to the respective treatments. The days from oviposition to individual eclosion was taken as development time. Body size was recorded using the surrogate of the radial cell length of the forewings [47], measured using ImageJ software.

Protein biochemical assay

Protein is important for immune function and host health [48,49], so amounts per individual were taken as one measure of host condition. At five-days post-eclosion, abdomens of isolated individuals were homogenized in 1 mL ringer solution. Using a Pierce™ BCA Protein Assay Kit, 200 μL of the working reagent was added to 25 μL samples in duplicate. Samples were incubated at 37°C for 30 min in darkness before absorbance was measured at 562 nm. Following blank subtraction, protein amounts per sample were calculated based on bovine serum albumin standards.

Infection prevalence, intensity, and *Nosema* spore production

At five-days post-eclosion, *N. bombi* spore loads (i.e., spores visible under 400x magnification) and the total infection intensity (quantified by qPCR) were assessed. Sampling at five-days post-eclosion ensured a fixed timepoint for assessing infection intensity across individuals. Abdomens were individually homogenized in 1 mL ringer saline solution. 10 μL of the homogenate was placed onto a FastRead 102 counting chamber and observed under phase contrast microscopy. Spores were counted and converted to total spores per individual.

To quantify total infection, including intracellular stages, DNA was extracted from 200 μL of each abdomen homogenate with an IBI Scientific Fecal DNA Kit following the manufacturer's protocol. For each sample, the DNA quality and concentration (260 and 280 absorbance) was measured using a NanoDrop spectrophotometer. Infection intensity was measured by qPCR on a QuantStudio^{TM} 3 Real-Time qPCR machine. Reactions used the Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (300 nM) added to established BOMBICAR primers (10 μM each), specific to *N. bombi* [50]. 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 [50,51]. Infection intensities were determined from a standard curve of known *N. bombi* pure spore quantities, which had been isolated using a BD FACSMelody™ Cell Sorter and DNA extracted as above. Mean infection intensities were taken from three technical replicates per sample. Where the QuantStudio^{TM} 3 software indicated unacceptable coefficients of variation across technical replicates and an outlier replicate could be identified, that outlier was removed. In the case of an unacceptably high coefficient of variation where the spread of replicates prevented reliable identification of an offending outlier, the sample was rerun with three new technical replicates.

Survival

Individuals not sampled at five-days for the prior assays were tracked daily for survival. Upon death, the date was recorded, and *Nosema* infection was documented by checking for spores as above.

Statistical analyses

Analyses were performed in R version 3.6.3 “Holding the Windsock” for Mac [52]. Mixed Effect Cox Proportional Hazard models were fit with the *coxme* package [53] and Linear and Generalized Linear Mixed Effect models with the *lme4* [54] and glmmTMB [55] packages. For each response variable, potential distributions were assessed for model fit and adherence to assumptions. Initial models were simplified by sequentially eliminating non-significant terms based on likelihood ratio tests (LRTs) and nested models were compared and selected using AICc [56]. The statistics for terms not in final models were taken from the step before their removal. Estimated marginal means and their confidence intervals for levels of model terms and post hoc Tukey contrasts were performed with the package *emmeans* [57]. For Preference Assays, linear mixed models were fit with time period, chlorothalonil treatment, and their interaction. Source colony and individual bee were included as random effects. For consumption of pollen pellets (log-transformed) and sugar water per microcolony, linear mixed models were used with fixed effects being *Nosema* and chlorothalonil treatments, and their interaction. Microcolony nested within source colony was included as a random effect to account for non-independent repeated measures. The analysis of developing larvae making it to adulthood was performed with a generalized linear mixed model with a binomial distribution and a logit link function, with the response being the number successfully emerging to adulthood to the number not. *Nosema*, chlorothalonil treatment, and their interaction were included with source colony as a random effect.

For all analyses on individuals produced from microcolonies, microcolony nested within original source colony was included as a random effect. For individual development, adult body size, protein level, and survival fixed effects were *Nosema*, chlorothalonil exposure, and their interaction. Additionally, when it was not the response variable, body size and all two and the three-way interactions were initially included. In addition, analyses only on *Nosema* exposed individuals replaced *Nosema* exposure with the status of infected or not infected, based on evidence from qPCR or spore checks. Linear mixed models were used for development, body size and protein levels. Survival was analyzed with a Mixed Effect Cox Proportional Hazards model.

Total infection intensity and transmissible spore data were analyzed in *Nosema* exposed bees with body size, chlorothalonil exposure, and their interaction as fixed effects. Infection prevalence was analyzed with a generalized linear mixed model with a binomial distribution and a logit link. To account for overdispersion, a generalized linear mixed model with a negative binomial distribution with a linear parameterization [58] and a log link function was used for infection intensity in those individuals identified as infected. In infected individuals, prevalence of spore production and spore counts were analyzed in the same way.

RESULTS

Chlorothalonil preference assays with adult bumblebees: no choice and choice

When provided with either chlorothalonil (n=19) or control (n=17) sugar water, bumblebee worker daily consumption did not significantly differ between treatments (Figure 1A, χ^2=0.215, df=1, p=0.643), nor the interaction involving time (χ^2=1.941, df=1, p=0.164). Similarly, when individuals (n=40) were given a choice of either chlorothalonil or control sugar water, consumption again was not influenced by chlorothalonil (Figure 1B, χ^2=0.413, df=1, p=0.52), nor its interaction with time (χ^2=0.188, df=1, p=0.665). There was a significant effect of time period, with daily consumption decreasing in the second time period for both no choice (χ^2=36.224, df=1, $p < 0.001$) and choice (χ^2=24.254, df=1, $p < 0.001$) assays.

Whole microcolony traits under chlorothalonil and *Nosema* treatments: consumption and progression of treated larvae to adulthood

Consumption within microcolonies was recorded at three successive timepoints (48, 96, and 144 h post-treatment initiation). Pollen consumption of microcolonies was not influenced by *Nosema* exposure (χ^2=0.002, df=1, p=0.969), chlorothalonil (χ^2=0.0004, df=1, p=0.985), their interaction (χ^2=1.884, df=1, p=0.17), nor the three-way interaction with time (χ^2=2.367, df=2, p=0.306). However, pollen consumption was affected significantly by time (χ^2=62.677, df=2, $p < 0.001$). Pollen consumption increased significantly at each timepoint (Tukey HSD $p < 0.05$).

Microcolony sugar water consumption was also not significantly influenced by *Nosema* (χ^2=1.08, df=1, p=0.298) or chlorothalonil (χ^2=0.353, df=1, p=0.553) exposures, their interaction (χ^2=0.056, df=1, p=0.814), nor the three-way interaction with time (χ^2=0.97, df=2, p=0.616). However, as with pollen, time significantly affected sugar water consumption (χ^2=10.562, df=2, p=0.005), which was driven by reduced consumption at 144h relative to 48h (Tukey HSD p=0.063) and 96h (Tukey HSD p=0.005) post-treatment initiation.

The probability of progression of individuals within microcolonies from early instar larvae at treatment initiation to adulthood was not significantly affected by *Nosema* (χ^2=2.492, df=1, p=0.114) or chlorothalonil (χ^2=0.219, df=1, p=0.640) treatments, nor their interaction (χ^2=1.733, df=1, p=0.188).

Individual traits of development time, adult body size, and protein

Across all treatments, individuals (n=481) reached adult eclosion on average 31.36 days post-oviposition. There was no effect of *Nosema* exposure (χ^2=0.026, df=1, p=0.871), chlorothalonil (χ^2=0.092, df=1, p=0.761), nor their interaction (χ^2=2.408, df=1, p=0.121) (Figure S2). However, analyzing those bees exposed to *Nosema*, based on their status of infected (n=155) or not infected (n=109), infection significantly affected development time (χ^2=5.707, df=1, p=0.017). On average, *Nosema* infected bees emerged as adults 16.27 hours sooner than those exposed but uninfected (Figure S3). In this subset, there was also no significant effect of chlorothalonil treatment (χ^2=0.646, df=1, p=0.422), nor the interaction of chlorothalonil treatment and *Nosema* status (χ^2=0.175, df=1, p=0.675).

Individual adult body size was not affected by *Nosema* (χ^2=0.625, df=1, p=0.429), chlorothalonil (χ^2=0.017, df=1, p=0.895), nor their interaction (χ^2=1.751, df=1, p=0.186) (Figure S4). However, again analyzing only *Nosema* exposed bees based on infection status, there was a significant effect of infection on body size (χ^2=4.751, df=1, p=0.029). Infected bees (forewing radial cell=2.82 mm) were on average 1.81% larger than those uninfected (2.77 mm). In this subset there was again no significant effect on body size of chlorothalonil (χ^2=0.71, df=1, p=0.399), nor the *Nosema* status by chlorothalonil interaction (χ^2=2.005, df=1, p=0.157) on body size.

Protein amounts in bees (n=113) did not differ based on exposure to *Nosema*, chlorothalonil, nor their interaction (Table S1; Figure S5). Unsurprisingly, protein amount was positively affected by body size. These results were consistent when considering infection status in only *N. bombi* exposed bees (n=57) (Table S2).

Infection outcomes: total infection intensity and spore production

Of *Nosema* exposed bees assayed by qPCR at five days post-eclosion (n=161), 58.75% showed evidence of infection. Infection data were therefore analyzed as the prevalence based on binary presence or absence of infection and also the total infection intensity in infected individuals. Neither chlorothalonil treatment (χ^2=1.413, df=1, p=0.235) nor the interaction of body size and chlorothalonil treatment (χ^2=0.094, df=1, p=0.759) affected the likelihood of a bee being infected. However, there was a significant effect of body size (χ^2=4.508, df=1, p=0.034), with larger bees being more likely to be infected (Figure S6). This mirrors the prior detected difference in size between infected and uninfected individuals. In infected bees (n=94), the total *Nosema* infection intensity was not influenced by chlorothalonil treatment (χ^2=1.377, df=1, p=0.242, Figure 2A), body size (χ^2=0.693, df=1, p=0.405), nor their interaction (χ^2=0.133, df=1, p=0.715).

Considering transmission potential, 69% of infected individuals had spores present at five-days post-eclosion. Spore presence was not influenced by chlorothalonil (χ^2=0.941, df=1, p=0.332), body size (χ^2=0.877, df=1, p=0.468), nor their interaction (χ^2=0.045, df=1, p=0.832). Where spores were present (n=65), there was an overall positive relationship between the quantified infection intensity and spore number ($F_{1,63}$=187.5, $p < 0.001$, R^2 =0.745). However, the number of spores present was significantly affected by chlorothalonil treatment (χ^2=7.311, df=1, p=0.006). Those bees exposed to chlorothalonil during development had a greater number of extracellular *N. bombi* spores than those bees not exposed to chlorothalonil (Figure 2B). There was no effect of body size (χ^2=0.976, df=1, p=0.323) or the chlorothalonil treatment and body size interaction (χ^2=1.155, df=1, p=0.283). Further, the percentage of the total quantified infection represented by transmission ready spores was significantly greater in chlorothalonil exposed bees (estimated marginal mean 4.71% [95% C.I.s: 3.98-5.43%]) versus unexposed (2.77% [1.90-3.63%]) (χ^2=12.197, df=1, p < 0.001). Body size did not significantly affect this proportion (χ^2=0.204, df=1, p=0.651), nor did its interaction with chlorothalonil (χ^2=0.124, df=1, p=0.724).

Adult survival

For individuals tracked for survival (n=253), there was no effect of exposure to *Nosema*, chlorothalonil, body size, nor all two- and three-way interactions on survival (Table S3A, Figure S7). In *Nosema* exposed individuals (n=101), survival hazards were higher in infected versus non-infected individuals, but this difference was not significant (Figure 3, Table S3B). Furthermore, neither chlorothalonil exposure nor any interactions involving body size significantly affected survival (Table S3B), but body size had a borderline-significant effect on survival (χ^2=3.834, df=1, p=0.050).

DISCUSSION

Pesticide and pathogen co-exposure has gained increasing attention in the literature regarding threats to native bee pollinators [6]. A main focus has been on neonicotinoid insecticides, but a landscape analysis identified chlorothalonil fungicide use to be associated with prevalence of the microsporidian *N. bombi* in declining North American *Bombus spp.* [36]. We demonstrate that *B. impatiens* workers do not avoid chlorothalonil-laced sugar water, and pollen and sugar water consumption in worker produced microcolonies does not change when provisions are spiked with field-realistic chlorothalonil doses (100 ppb). We did not see a preference, as reported for 50 ppb chlorothalonil in honeybees [37], but a lack of avoidance suggests that foraging bumblebee workers are unable to detect or avoid sublethal chlorothalonil concentrations. This sets the stage for co-exposure to the potential multiple stressors of this fungicide and pathogens such as *N. bombi* and emphasizes the importance of studies investigating the effects of their co-exposure.

Testing the multiple stressor hypothesis in worker produced *B. impatiens* microcolonies exposed to chlorothalonil or *N. bombi* early in development, we found no strong evidence for the hypothesis in relation to tolerance or resistance of individual hosts to infection. Development, size, survival, and protein amounts of males from microcolonies were not significantly negatively affected by *Nosema* exposure or infection*,* chlorothalonil exposure, nor their interaction. Additionally, the prevalence and infection intensities at five-days post-eclosion did not differ. This suggests that, under our experimental set up and measured traits, chlorothalonil co-exposure does not affect individual resistance to infection or health outcomes, as has been demonstrated in other pathogen-pesticide experiments on bees [e.g., 12]. As highlighted earlier, it is important to note that our study species is considered stable [16], and thus is not one of the declining species in which prevalence of *N. bombi* has been associated with local chlorothalonil use [36]. However, despite this caveat, we show that an infection outcome relating to subsequent transmission potential of the pathogen is affected. Although total infection intensities did not differ, total spore production from established infections was altered by chlorothalonil treatment. Bees from microcolonies exposed to chlorothalonil exhibited increased spore loads, with spores representing a greater proportion of the total infection intensity. This indicates that in bumblebees, chlorothalonil exposure can interact with *N. bombi* infection to influence a parameter important for transmission dynamics that could affect colony, population, or community health. This could, in part, explain elevated infections, or at least facilitation of infection, in declining species [16,36].

We did not document any individual negative effects of chlorothalonil exposure or *N. bombi* exposure or infection. This contrasts with other studies, where dietary exposure of bumblebees to other pesticides frequently reduces traits associated with individual or colony fitness [11–15,60–62]. Chlorothalonil has not been as widely studied as neonicotinoids, but negative effects have been found in honeybees [40], including effects of treatment concentrations 10-fold lower than used for our microcolony experiment [39]. Another study in *B. impatiens* showed that colonies produce fewer workers, less biomass, and have smaller queens following chlorothalonil exposure [38]. The exact consumed doses cannot be compared, due to the mode of application, but the dose was likely higher in this earlier study. Thus dose, exposure time, microcolony conditions, or caste differences could all be explanations for a lack of similar negative effects in our study. Although the concentration of chlorothalonil we used in exposures likely represents a reasonable approximation of an average dietary field exposure [35,44], exposures could be much higher [44]. Subsequent studies are required to test if such high field realistic doses could have stronger negative individual or pathogen-associated interactive effects.

The absence of negative *N. bombi* effects are perhaps more surprising than for chlorothalonil, given the documented detrimental effects of infection [25,26]. Although survival hazards suggested that *Nosema* infected bees had a greater risk of death, this was not significant, nor were there any other apparent reductions in individual health measures. In fact, exposed and infected bees developed slightly faster and were slightly larger than exposed uninfected bees. The relevance for host or pathogen and the cause of these effects is not clear, but they could potentially stem from a cost of successfully resisting infection [63]. An alternative to resistance of pathogen infection is tolerance, the withstanding of negative effects of a particular infection level [64]. No previous studies of the effects of *N. bombi* have been carried out in *B. impatiens*, but a lack of detrimental effects, even when individuals were carrying high infections, suggests that *B. impatiens* is either better able to tolerate infection or virulence outcomes are context dependent, as with other bumblebee pathogens [59,65]. Investigating context dependence and particularly species differences in resistance and tolerance, including in declining species, is an important future research avenue [6].

Between-host dynamics of pathogens and contributing factors, such as transmission potential, are key determinants in the epidemiological spread and the impact on host populations and communities [66–69]. While within- and between-host dynamics of infection are unavoidably interrelated [66], the presence of specific transmission stages, such as the extracellular spores of *N. bombi*, can decouple them to some extent. We show that chlorothalonil exposure did not alter total infection intensities but did result in greater spore loads. Spore production can be equated to transmission potential, indicating that chlorothalonil exposure has the potential to alter the association between within- and between-host dynamics. Subsequent increased disease spread in colonies, and host populations or communities, through contamination of floral resources [70], would be predicted to result in higher overall pathogen prevalence and loads at these ecological scales under basic epidemiological models [71]. Even if some hosts exhibit high infection tolerance, as we see in this study, there will be an increased likelihood of transmission to more susceptible individuals or species, where *Nosema* alone may exhibit its documented high virulence [25,26] or chlorothalonil co-exposure may affect within-host total infection levels or their consequences. These represent plausible links between the demonstrated effect of chlorothalonil on *N. bombi* infection outcomes and the landscape-level association reported by [36]. This should prompt further studies under the multiple stressor framework into this pesticide-pathogen interaction, including between-host dynamics.

Potential mechanistic explanations for the effect of chlorothalonil on *N. bombi* spore production may be through a disruption of host physiology and immunity, alterations to the host gut microbiota, or potentially terminal investment strategies of the microsporidian. In honeybees, chlorothalonil exposure significantly enhanced glucose oxidase activity [39], a marker for social immunity. Although, resistance to infection was not obviously compromised in our study, host immune or other physiological changes could alter spore production dynamics. Furthermore, honeybees and bumblebees have gut microbiota that can determine infection outcomes [72], and chlorothalonil has been shown to change honeybee gut microbiota structural and functional properties [73]. Chlorothalonil could indirectly enhance spore production by disturbing the bumblebee gut microbe community. Finally, increased spore production may result from a pathogen terminal investment strategy under stress [74]. When host conditions are unfavorable, pathogens are predicted to switch strategies from within-host replication to transmission stages [75], including in response to anti-pathogen treatments [e.g., 76]. Molecular phylogenetic data suggest that microsporidia are either a basal branch of the Fungi or a sister group [77]. Thus, fungicide exposure could precipitate such a strategy shift in *Nosema.* However, we do not see reductions in prevalence and infection loads that we would expect if chlorothalonil negatively affected *N. bombi*, making direct or indirect effects on the host more plausible.

Understanding and preventing diseases outbreaks that threaten biodiversity and ecosystem services requires investigations of patterns and causation. Our study builds on prior demonstrations of an association between chlorothalonil use and prevalence of *N. bombi* in declining North American bumblebees [36]. Using experimental exposures in worker produced microcolonies of the non-declining *B. impatiens*, we demonstrate that co-exposure to field-realistic concentrations of chlorothalonil can increase *N. bombi* spore production and thus transmission potential. This demonstrates the need to consider factors relating to both within- and between-host dynamics of infection when considering pathogens under the multiple stressor hypothesis framework.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

A.C.C. conducted the experimental work and data collection, participated in data analysis, wrote the first draft of the manuscript, and collaborated on experimental design; B.M.S. collaborated on experimental design, performed data analysis, and revised the manuscript; A.E.H. assisted in experimental set-up; A.E.H. and T.A.B. assisted in data collection and manuscript review.

ETHICS, CODE AND MATERIALS

Data and scripts available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.w3r2280pg.

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FIGURE LEGENDS

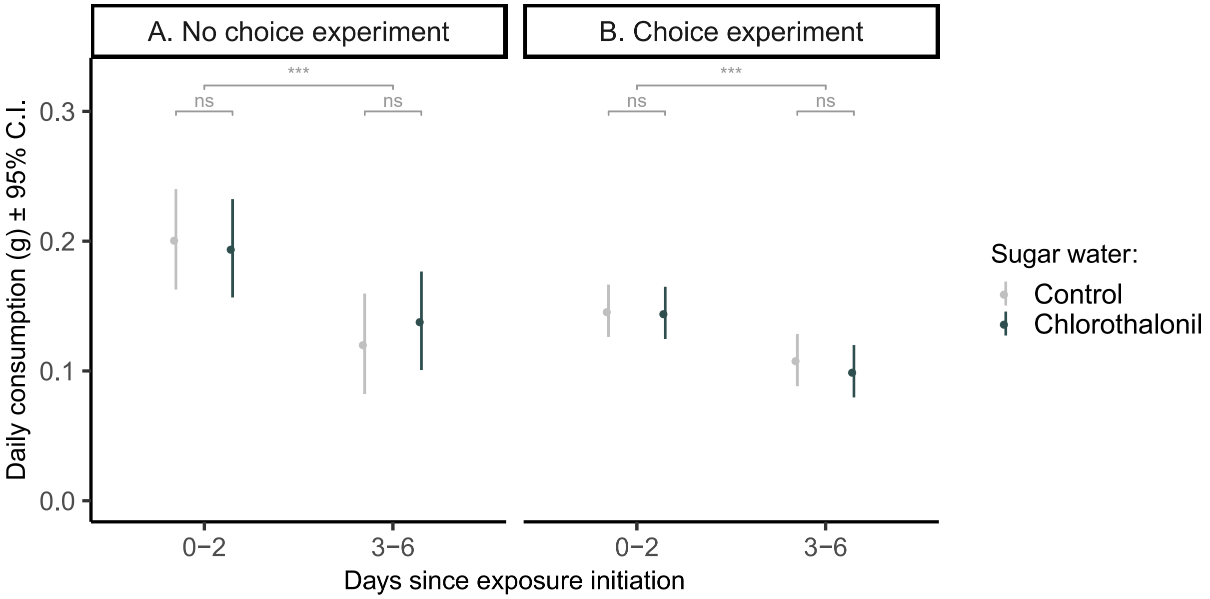

Figure 1. Daily consumption (g) of treated sugar water by adult bumblebee workers in a no choice preference assay (A) and in a choice preference assay experiment (B) during the periods of zero to two days and three to six days after treatment initiation. Individual bees are represented by small points around the estimated marginal mean, bars represent 95% confidence intervals. Brackets indicate nonsignificant (ns) or significant pairwise comparisons (***p<0.001).

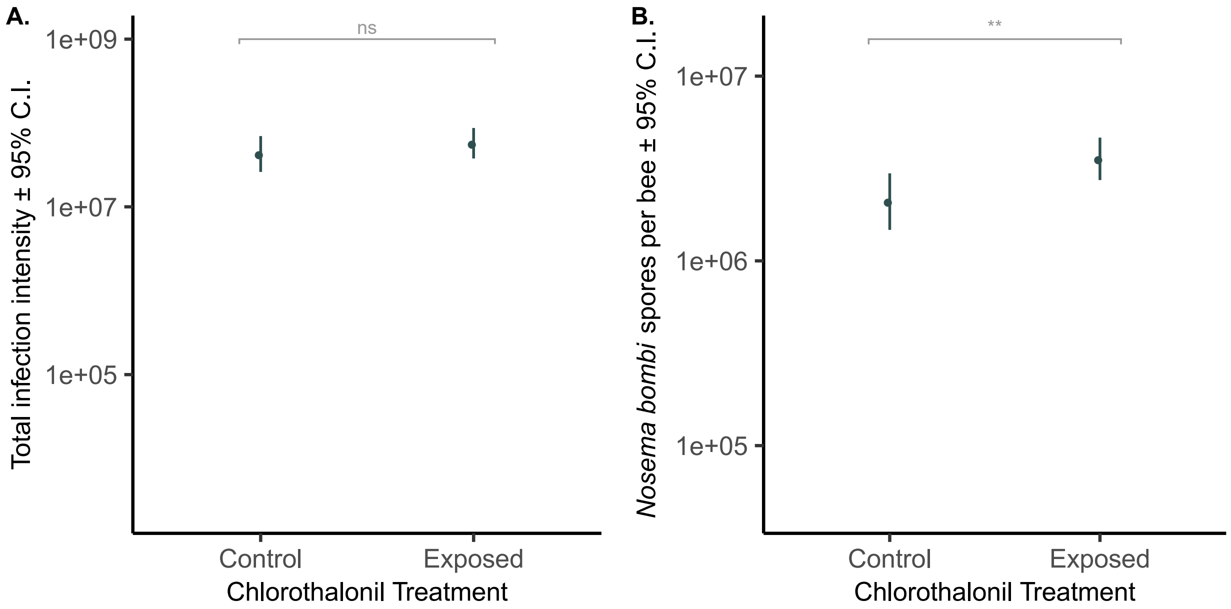

Figure 2. The effect of chlorothalonil treatment on (A) total infection intensity and (B) *N. bombi* transmission-ready spores counts. Total *N. bombi* infection intensity is based on qPCR and spores from counts at five days post adult eclosion, for bees exposed to chlorothalonil or not during development. Y-axes $\log_{10}$-transformed. Individual bees are represented by small points around the estimated marginal mean, bars represent 95% confidence intervals. Brackets indicate nonsignificant (ns) or significant pairwise comparisons (**p<0.01).

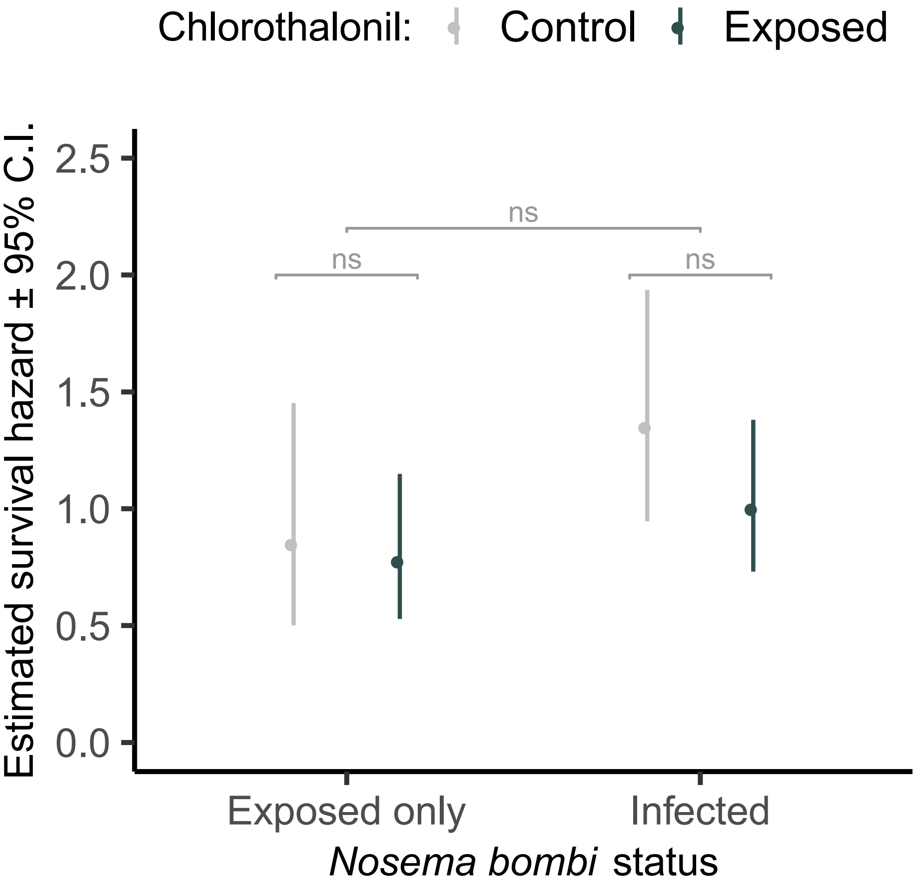

Figure 3. Estimated survival hazard of bees based on *N. bombi* status and chlorothalonil exposure. Points represent model estimated survival hazard values and error bars represent 95% confidence intervals. Brackets indicate nonsignificant (ns) pairwise comparisons.