Past, present, and future: Non-consumptive effects of the ectoparasitic mite *Macrocheles subbadius* on adult and larval *Drosophila nigrospiracula*

By

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Abstract

Parasites can indirectly impact hosts through non-consumptive effects (NCEs) via changes in behaviour, morphology, and/or physiology. These responses can be understood in terms of the ecology of fear framework. My study system involves a cactophilic fruit fly (*Drosophila nigrospiracula*) and a naturally occurring ectoparasitic mite (*Macrocheles subbadius*). Previous studies have shown that chronic mite exposure (without infection) in adult flies decreases fecundity and longevity, although the mechanism leading to that decrease is unknown. I tested the hypothesis that NCEs of parasite exposure (e.g., parasite avoidance and defense) trade off with fitness-related behaviours such as feeding and resting. I also posited that the magnitude of these NCEs would be amplified by either primary exposure (*sans* infection) or primary infection history. I found that secondary mite exposure (through free roaming mites in an observation arena) resulted in increased grooming and movement, but exposure history did not affect these behaviours. However, the interaction between primary and secondary exposure influenced host feeding and resting behaviours. Upon a secondary mite exposure, previously exposed flies increased feeding and decreased resting, suggesting an important role for exposure history in the expression of NCEs. I then tested the role of infection history on the NCEs of current parasite exposure. Regardless of prior infection status, flies increased defensive and ambulatory behaviour in the presence of mites, and consequently less time was spent resting but feeding was unaffected. None of the behaviours measured were affected by previous infection status. Moreover, these results showed that previous exposure (*sans* infection) to parasites may have an even stronger effect upon secondary exposure than infection history. Secondly, I investigated whether *M. subbadius* exert non-consumptive effects on fly larvae that persist through

development. Even though mites do not infect larvae, previous work has shown that mite presence exerts NCEs by reducing pupation success. I hypothesized that exposure to mites during the larval stage has downstream effects, such that the NCEs persist through development into the adult stage. I predict that larvae exposed to mites will exhibit decreased body weight, fecundity, and longevity as adults. The results showed no evidence of a downstream effect on body weight, fecundity, or longevity. Since parasites were absent in adulthood, perhaps adult flies did not prioritize parasite defence under conditions of reduced risk. NCEs of parasite exposure on larvae did not carry-over into the next life stage adulthood. My study highlights the importance of the ecology of fear and the role that parasites play in generating non-consumptive effects of parasitism. The ecology of fear should be integrated into management decisions as parasites not only modify current host behaviour, but have the potential to affect future behaviours, even without active infection.

Preface

All chapters are original work done by Caroline Liang

Chapter 1 is the general introduction and objectives.

Chapter 2 is an adaptation of "Ghosts of parasites past influence current non-consumptive effects in *Drosophila nigrospiracula*". Currently in review in the *International Journal of Parasitology* (2024), co-authored by C. Liang and L.T. Luong. I designed and conducted the experiment, analysed the data, and wrote the manuscript. L.T. Luong assisted in refining the protocol and contributed edits to the manuscript.

Chapter 3 is original work done by C. Liang.

Chapter 4 is the general conclusion with future directions.

Dedication

To my support system: 妈妈, 爸爸, Helena, and Scott for helping me push through despite the stress, 谢谢！ This thesis is dedicated to each of you as it would not have been possible without your love and daily encouragement.

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Chapter 1: General introduction

1.1 Overview and background

Predators regulate prey populations through the direct killing of prey, thus decreasing prey density (Gasaway et al., 1992; Sih et al., 1992). However, there is evidence that predators influence prey dynamics through their presence alone, and those changes are described as "non-consumptive effects" (NCEs). Predators can even increase plant density by decreasing herbivore densities through the direct killing and through altered herbivore foraging and habitat use (Beckerman et al., 1997; Fortin et al., 2005). For example, the presence of spiders, even with their mouthparts experimentally glued shut, decreased herbivory in grasshoppers (Beckerman et al., 1997). The presence of predators can induce changes in prey habitat selection, diet, and timing of activity, even without active predation (Beckerman et al., 1997; Fortin et al., 2005; Hawlena and Pérez-Mellado, 2009; Hawlena and Schmitz, 2010a; Iwasa, 1982; Kotler, 1984; Lima, 1998). Unlike direct predation, predator exposure can be chronic, and this can result in sustained stress in organisms (Clinchy et al., 2013). NCEs of predation can be long-lasting: predator cues decreased song sparrow (*Melospiza melodia*) clutch size and disrupted incubation, leading to lower hatch success, decreased parental foraging, and greater nestling starvation (Zanette et al., 2006).

Parasites are ubiquitous in the environment: outnumbering predators and with greater diversity, including numerous parasites species yet to be described (Dobson et al., 2008; Okamura et al., 2018). They also possess higher total biomass and can impose stronger selective pressures on their hosts compared to predators, and in some cases, they can regulate host densities (Dobson et al., 2008; Kuris et al., 2008; Weinstein et al., 2018a). Parasites are deeply entrenched in food webs, representing 75% of all links in food webs (Dobson et al., 2008; Windsor, 1998). Parasite infection causes physiological damage, reduced dispersal capacity, can mechanically interfere with reproduction and feeding, and represents an energetic burden, (Lezama-Davila et al., 2013; Luong et al., 2015; Polak, 1996). As a result, infection reduces growth rates, decreases body mass and increases mortality (Fjelldal et al., 2020; Lezama-Davila et al., 2013; Lynsdale et al., 2017, 2017). On the extreme end, certain parasites can reduce fitness to zero, by castrating hosts (Lafferty and Kuris, 2009). Parasite infection must be avoided to

prevent losses in fecundity, reproductive success, and decreases in offspring quality (Booth et al., 1997; Ebert, 1995; Gooderham and Schulte-Hostedde, 2011; Hasik and Siepielski, 2022; Polak, 1996; Schwanz, 2008).

There is growing evidence that exposure to parasites, even without infection, can impose costs on potential hosts (Gibson and Amoroso, 2022; Giorgi et al., 2001; Horn and Luong, 2018; Luong et al., 2017; Ower and Juliano, 2019). Potential hosts in the presence of their parasite may decrease foraging and general activities (Fritzsche and Allan, 2012; Orr, 1992; Philpott et al., 2004; Rohr et al., 2008; Selbach et al., 2022; Weinstein et al., 2018b). For example, the presence of parasitic phorid flies (*Pseudoacteon* spp.) decreased ant (*Azteca instabilis* F. Smith) predation on a coffee pest, the coffee berry borer (*Hypothenemus hampei* Ferrari), resulting in economic losses (Pardee and Philpott, 2011). Like predators, NCEs of parasites can have cascading effects on trophic chains. Parasites can also reduce longevity and fecundity through indirect contact in a fly-mite association (Horn and Luong, 2018). However, less is known about the NCEs of parasitism relative to that of predation.

The study system used for this thesis involves species found in the Sonoran Desert (Arizona, USA): the cactophilic fly *Drosophila nigrospiracula* and its parasite, *Macrocheles subbadius*. *D. nigrospiracula* consumes decaying tissues of two species of columnar cacti, *Carnegiea gigantea* (saguaro cactus) and *Pachycereus pringlei* (cardón cactus) (Martinson et al., 2017). These flies are specialized in feeding on these cacti: cactus decomposition volatiles recruit *D. nigrospiracula* but exclude other cactophilic *Drosophila* species (Heed, 1978; Perez-Leanos et al., 2017). Larval and adult *D. nigrospiracula* possess cytochrome P-450 monooxygenase enzymes that allow them to metabolize and tolerate otherwise toxic plant allelochemicals, such as the alkaloids in saguaro (Danielson et al., 1994).

D. nigrospiracula is capable of long-distance dispersal, contributing to minimal genetic drift (Johnston and Heed, 1976; Markow and Castrezana, 2000). Fly dispersal is heightened under certain conditions: in the evening, under elevated temperatures, when cactus densities are low, and when the cactus necroses dry out (Johnston and Heed, 1976). The age of cactus necrosis is correlated with the risk of parasitism: with mite density and infection prevalence increasing with the age of cactus necrosis (Polak and Markow, 1995). Necrotic cactus tissue serves as an ephemeral habitat with rot persisting from 1 week to 3 months. Flies exhibit reduced dispersal during the initial stages of the rot, and exhibit increased dispersal with decreasing nutrient levels

and increasing mite densities as the necrotic tissue undergoes drying (Johnston and Heed, 1976; Polak and Markow, 1995). Females are mostly found on streams of cactus exudate while males were more abundant in the healthy portions of the cactus (Markow, 1988). Males occupy restricted "territories" and females visit male territory for mating (Markow, 1988). *D. nigrospiracula* reaches sexual maturity at 10 days for females, and 4-5 days for males (Markow, 1988; Polak and Starmer, 1998). Females tend to oviposit on necrotic cactus tissue during its early stages of rot, and it is more nutritious compared to drying cactus (Fogleman et al., 1981; Johnston and Heed, 1976; Markow, 1988).

Macrocheles subbadius is an ectoparasite of *D. nigrospiracula*. These mites consume fly hemolymph and damage fly cuticle (Polak, 1996). These mites exhibit behaviours akin to invertebrate predators: they explore the environment, and upon nearing a fly, they reduce ambulatory speed, suggesting the ability to detect host-related stimuli and increasing infection success (Perez-Leanos et al., 2017). *M. subbadius* prefers to infect *Drosophila* of the *repleta* species group. They may recognize species through epicuticular hydrocarbon profiles, and that may contribute to infection preference (Markow and Toolson, 1990). Within a host species, mites also preferentially infect flies with higher respiration rates and larger body sizes (Horn et al., 2018, 2023a), suggesting a trade-off between host body size and infection risk. An infection amplification loop is found in this fly-mite association: infection status increases the likelihood of acquiring more infections (Brophy and Luong, 2022). Therefore, preventing the initial mite infection is vital for host fitness, emphasizing the need to for increased avoidance behaviours. Behavioural strategies to reduce the risk of ectoparasitism include grooming, tarsal flicking, preening, and scratching (Benoit et al., 2020; Clayton et al., 2010; Giorgi et al., 2001; Hart and Hart, 2018; Sarabian et al., 2018; Zhukovskaya et al., 2013). While behavioural changes may incur costs, their potential to prevent infection may outweighs these costs.

1.2 Research Objectives

Although Horn and Luong (Horn and Luong, 2018) showed that chronic mite exposure (*sans* infection) decreased fitness, the underlying mechanism is unclear. In chapter 2, I aimed to identify: 1) behavioural trade-offs between NCEs and other host behaviours, 2) the effect of mite exposure history (without infection) on the expression and magnitude of NCEs, and 3) the impact of infection history on the expression and magnitude of NCEs. I examined how time

allocation to behaviours changed with mite presence and how exposure and infection history affected these changes. In the first experiment, I tested the hypothesis that increased parasite NCEs such as avoidance behaviours (grooming) trade off with host feeding. Grooming is a time- and energy-intensive behaviour (Giorgi et al., 2001; Zhukovskaya et al., 2013). I used a 2x2 factorial set up with different levels of exposure history (primary exposure) and secondary exposure to test the hypothesis that organisms can learn (via previous experience) to avoid parasite cues and subsequently show stronger avoidance responses upon secondary mite exposure. Behaviours measured included grooming, moving, feeding, and resting. In the second experiment, we varied primary infection status instead. We hypothesized that a history of infection further increases the expression and magnitude of NCEs upon secondary exposure. An experienced fly (previously exposed and infected) should mount stronger defensive behaviours during a secondary encounter with mites. We measured grooming, ambulation, tarsal flicking, bursts of flight, feeding, and resting.

There is yet another possible mechanism for decreased survival and fecundity among adult female flies under chronic mite exposure (Horn and Luong, 2018). NCEs of parasitism could affect larval development despite this life stage being non-susceptible to these ectoparasites. In chapter 3, I aimed to elucidate whether fly larvae exposed to mites show NCEs that carry-over to the adult stage. Previous research has shown that larvae exposed to mites have decreased pupation success and larvae pupate away from mite cues when possible, however it is unclear whether these NCEs would affect adult phenotype (Horn et al., 2023b). Parasite exposure can result in accelerated development, and smaller body sizes (Ower and Juliano, 2019). Smaller body sizes can then reduce fecundity (Lefranc and Bundgaard, 2000). I hypothesized that larvae exposed to mite cues will exhibit NCEs that carry over into the adult stage, resulting in decreased body condition and fitness. I exposed second instar larvae to caged mites, allowed the larvae to progress through development, then recorded adult emergence weight, fecundity, and longevity.

1.3 Significance

Understanding the mechanisms underlying fitness losses in the presence of ectoparasites, even in the absence of infection, is important given the widespread presence of parasites in the environment. Additionally, finding out whether parasites can affect non-susceptible stages, such as larvae, demonstrates the scope of the ecology of fear associated with parasites. My thesis

investigates both short- and long-term impacts of parasites on host organisms. The ecology of fear operates over short and broad time scales: hosts may alter their behaviour to avoid immediate infection upon encountering parasites, and those changes may result in long-term effects. Repeated or chronic mite exposure can lead to lasting changes in fitness or life history traits.

Chapter 2: Ghosts of parasites past influence current non-consumptive effects in *Drosophila nigrospiracula*

2.1 Introduction

Predators exert pressure on their prey both directly (via consumption) and indirectly, whereby the presence of predators alone can decrease prey populations (Preisser and Bolnick 2008). For example, mule deer show higher vigilance and spend less time foraging if they sense a predator nearby (Altendorf et al. 2001). These 'non-consumptive' effects (NCEs) include changes in prey behaviour, morphology, and physiology (Pijanowska, 1992; Brown et al., 1999; Kollross et al., 2023). Potential behavioural changes such as altered foraging and increased emigration can in turn decrease prey density with the potential for cascading effects on the food web (Preisser et al., 2005; Preisser and Bolnick, 2008; Kollross et al., 2023). Predatory cues may also induce reallocation of resources to other traits associated with predator avoidance and escape (Kohler and McPeek, 1989; Herberholz and Marquart, 2012; McMahon et al., 2018; Kollross et al., 2023). This 'ecology of fear' can be adaptive but can also trade off with has the growth, survival, or reproduction, which can in turn reduce prey population growth (Pijanowska, 1992; Peacor and Werner, 2008; Preisser and Bolnick, 2008).

It is well documented that parasites cause harm through direct infection (Poulin and Thomas, 1999; Sears et al., 2013, 2011; Luong et al., 2017, 2015), but the presence of parasite or parasite cues can also induce NCEs in hosts (Buck et al., 2018). For example, tadpoles avoid predator and parasite cues to similar degrees (Rohr et al. 2008), suggesting that both natural enemies produce a similar avoidance response. Parasite avoidance is the first line of defense; different strategies of spatial and temporal avoidance, and selective foraging reduce the risk of infection (Orr, 1992; Hutchings et al., 2002, 2007; Rohr et al., 2008; de Roode and Lefèvre, 2012; Daversa et al., 2018, 2021b). Organisms can groom to remove parasites, avoid parasite-laden food and habitats, avoid infected conspecifics, and/or change foraging time to avoid peaks in parasite activity (Orr, 1992; Hutchings et al., 2002, 2007; Rohr et al., 2008; de Roode and Lefèvre, 2012; Zhukovskaya et al., 2013). These avoidance behaviours can indirectly lead to nutritional deficiencies, for example sheep feed less in the presence of parasites and tend to forage in parasite-free pastures, but of lower quality (Hutchings et al. 2007). Similarly, mammal hosts more readily abandoned heavily tick-infested foraging sites, presumably because foraging benefits were outweighed by

the costs of parasitism (Fritzsche and Allan, 2012). These studies demonstrate that NCEs have been observed across a range of taxa, yet there are relatively few empirical studies addressing the ecology of fear or disgust in parasite-host relationships.

A particularly tractable system for investigating the ecology of fear in a host-parasite association is found in the Sonoran Desert (Arizona, USA). The cactophilic fly *Drosophila nigrospiracula* (Diptera: Drosophilidae) reproduces on rotting saguaro cactus (*Carnegiea gigantea*) (Markow, 1988) and is naturally associated with the ectoparasitic mite *Macrocheles subbadius* (Acari: Macrochelidae), which uses various flying dipterans as transport to resources such as necrotic plant tissues (Polak and Markow, 1995). Only female mites are infective and feed off their hosts (Polak 1993; Polak and Markow, 1995). Mites preferentially infect female flies over male flies showing that the risk of parasitism is greater for female flies (Horn et al., 2020). Mite infection is costly to fly survival, flight endurance, and reproductive success (Polak and Markow, 1995; Polak and Starmer, 1998; Luong et al., 2015).

Even without infection, parasite presence is harmful; mite exposure without infection has been shown to reduce fly lifespan and fecundity (Horn and Luong, 2018; Horn et al., 2023). However, the underlying mechanisms leading to these decreases are unclear. Exposure to mites elicits a variety of pre-attachment defensive behaviours in flies, including avoidance of mite-laden habitats, vigorous grooming, continuous locomotion and evasion, short bursts of flight, and reflex movements such as tarsal flicking (Giorgi et al., 2001; Polak, 2003; Zhukovskaya et al., 2013; Luong et al., 2017; Polak et al., 2023). Many of these behaviours are time-intensive and physiologically costly. Decreases in lifespan and fecundity, as previously mentioned, could be the result of altered time budgets. Defensive behaviours such as grooming may trade off with time and energy required for other important fitness-related activities such as feeding and resting (Stearns, 1989; Giorgi et al., 2001; Luong et al., 2017; Horn and Luong, 2021).

Trade-off theory states that when organisms invest in certain traits it may come at a cost to other beneficial traits due to limited resources (e.g. energy and time) (Stearns, 1989). In a first experiment, we first hypothesized that increased parasite avoidance behaviours, such as grooming, trade off with host feeding, i.e., less time spent feeding. Further, we aimed to assess whether the expression and magnitude of this trade-off depends on the history of exposure to mites (without infection). Previous studies have shown that a host's response to parasitism is state-dependent (Daversa et al., 2021a; Selbach et al., 2022); for instance previous exposure

(without infection) to trematodes led to reduced activity in mussels (Selbach et al., 2022). Accordingly, we also hypothesized that organisms can potentially learn to avoid parasite cues and show stronger avoidance behaviours during subsequent encounters (Klemme and Karvonen, 2018). We predicted that flies previously exposed to mites (primary exposure) will groom at higher frequencies and consequently feed at lower frequencies compared to naïve (inexperienced) flies during secondary exposure.

In a second experiment, we tested the hypothesis that the history of infection (primary infection) further increases the expression and magnitude of NCEs in subsequent exposures. Since mites were allowed to roam free and infect flies in this experiment, flies should mount stronger and potentially more costly defensive behaviours in subsequent encounters with mites. We predicted that compared to naïve flies, previously infected flies, even post-clearance, will exhibit increased behavioural defences such as grooming, tarsal flicking, continuous locomotion, and bursts of flight during secondary exposure. A fly that had been exposed *and* infected by mites should mount a stronger response during the secondary exposure than naïve flies and flies with a history of exposure only (without infection). The elevated expression of these behavioural responses is expected to come at the expense of time spent feeding and resting.

2.2 Materials and methods

2.2.1 Experimental animals

Drosophila nigrospiracula (Diptera: Drosophilidae) were collected from necrotic saguaro cacti in the Sonoran Desert (Arizona, USA) and used to establish laboratory cultures. Flies were reared in 200 mL bottles in media with instant mashed potato flakes, *Drosophila* medium (Formula 4–24 Instant Drosophila Medium, Carolina Biological Supply Company, Burlington, NC, USA), nutritional yeast, and 6 grams of autoclaved necrotic saguaro cactus (*Carnegiea gigantea*). Fly cultures were maintained in an incubator (12 h light, 25 °C: 12 h dark, 24 °C, 70% RH). *Macrocheles subbadius* (Acari: Macrochelidae) Berlese were collected from infected flies in the Sonoran Desert and used to start laboratory cultures. Mites were reared in a 2.5:1 mix of wheat bran and aspen wood shavings, with free-living nematodes and nutritional yeast. Mite cultures were kept separate from fly cultures in a separate incubator (12:12 L:D light cycle, 25°C, 70% RH).

2.2.2 Exposure history experiment

To test for the effects of mite exposure history, adult female (0 - 1 day old) flies were assigned to one of two treatment groups: 1) not exposed to mites (i.e., 'naïve') and 2) exposed to mites (i.e., 'experienced' or 'exposed without contact'). For both control and experimental treatments, flies were collected at emergence from the base culture and grouped into vials of 20 flies. Three days post-eclosion, flies in the experienced group were exposed to mites for 5 days in an agar vial containing a mite cage with 5 female mites (i.e., primary exposure). Mites were extracted from the cultures on the day of the experiment using a Berlese funnel, and only female mites were used. The mite cage consisted of a modified 2mL microcentrifuge tube (40 mm in length x 10 mm in diameter). Both ends of the tube were cropped and sealed with a piece of mesh (80-μm pore size) to allow airflow and detection of the mites and their cues without any physical interaction with the flies (Luong et al., 2017). Naïve flies were housed for 5 days in an agar vial with an empty mite cage. Afterwards, flies were housed (with a soaked cotton ball to prevent desiccation) without mites and starved for 4 days before commencing the secondary exposure assay (at 12 days of age); starvation encouraged feeding. For behavioural observations, we set up fly arenas (60 mm borosilicate glass Petri dish) consisting of a food patch (0.2 g necrotic cactus tissue and yeast in a microcentrifuge tube lid). A single fly (now 12 days old) from the control or experienced group was placed in the arena along with 7 free-roaming mites or no mites. The space in the Petri dish allowed mites to interact with the fly but flies were able to escape infection. No flies were infected in the arena. The presence or absence of behaviours (grooming, feeding, moving, and resting) was recorded by direct observation (eyesight) for an hour for each trial (n = 30). To prevent bias, the order in which the treatments were viewed changed with each observation period. Feeding was marked by observing fly proboscis extensions and head bobbing on a food patch (Wong et al., 2009). Immobility of the fly during a behavioural scan was marked as "resting". Resting was measured since reductions in resting can have adverse effects on immune function, learning, but it increases predator avoidance (Evans and Schmidt, 1990; Bryant et al., 2004; Seugnet et al., 2011; Hill et al., 2018).

2.2.3 Infection history experiment

This experiment was similar to experiment 1 except that the treatment involved previously *infected* flies. At emergence, female flies were extracted from culture bottles and put into agar

vials (20-22 flies/vial). Then, 20 3-day old flies were put into infection jars (115 mL volume glass jar filled with medium containing mites from the mass culture). Naïve flies were put into jars filled with mite-free medium (2.5:1 mix of wheat bran and aspen wood shavings, water, and yeast). A 0.5 cm wide space along the length of the infection jars was excavated to allow flies to roam within that space and interact with mites. The jar was covered with a plastic lid with a hole cut out and stoppered with cotton to allow flies to be added and removed from the jar. This set up allowed flies and mites to interact in a semi-natural space that mimics the necrotic pockets of a saguaro cactus. After one hour, flies were removed and anesthetized with CO_2. Infected flies were placed in an agar vial for an hour to standardize mite attachment time. The attachment period was limited to one hour to reduce the physiological costs of infection (e.g., host energy drain and tissue damage) while allowing flies to experience direct contact with the ectoparasites (i.e., primary infection). Flies had space to groom and remove their mites in the vials. After one hour, flies with 1-2 mites still attached were kept for further manipulations. These flies were lightly anesthetized, which caused most mites to fall off; remaining mites (attached) were carefully removed with a paint brush. Not all flies were infected, which could be a result of differences in resistance, but to minimize the effects of infection, these "previously infected" flies were then put into a new agar vial and left to recover for 2 days from physiological stress of infection. Naïve flies were handled the same way: they were anesthetized and stroked with a paintbrush. Once recovered, flies were starved for 3 days; a wet cotton ball was provided to prevent desiccation. Behavioural assays (secondary exposure) were then conducted in 60 mm borosilicate glass Petri dishes, each containing a food patch (0.2 g necrotic cactus and yeast in a microcentrifuge tube lid). A single female fly was placed in a dish with either 7 free-roaming mites or no mites. Flies were allowed to acclimate to the arena for 10 minutes before the commencement of data collection. Despite being anesthetized, no flies were infected by mites in the arena. Behavioural scans were performed visually every minute point for an hour for each tested fly (n=30 trials, 1 trial per fly) for the occurrence of feeding, grooming, resting, walking, jumping, and tarsal flicking. Specific categories of movement: jumping, tarsal flicking, and ambulation were scored in this experiment. Since flies only performed bursts of flight, an energetically expensive behaviour, in the presence of a parasite to resist infection (Polak et al., 2023), we only compared the effect of exposure history among flies exposed to mites in the secondary exposure.

2.2.4 Statistical analyses

Grooming, feeding, resting, and ambulatory activity were counted and analyzed using the R Statistical Program (R Core Team 2022). Analyses for the second experiment also included jumping and tarsal flicking counts. Generalized linear mixed models (GLMM, lme4 package, MASS package) were used to analyze the effects of the independent variables of primary exposure (experiment 1) or infection (experiment 2) (i.e., exposure or infection history) and secondary exposure (i.e., presence/absence of mites in the arena), and their interactions on response variables: grooming, feeding, ambulation, resting, jumping (experiment 2 only), and tarsal flicking (experiment 2 only) counts. Due to overdispersion, a negative binomial distribution was fitted to the models, except in experiment 1, where feeding was analysed with a quasi-Poisson distribution, and in experiment 2, tarsal flicking was analysed with a Poisson distribution (R Core Team 2022). If primary and secondary mite exposure showed an interaction, then separate GLMs were performed for naïve and experienced flies to analyze the simple effects. Random variables in the model included the date of observation and time of day (block). Stepwise model selection was performed (ANOVA), variables were dropped if there was no significant difference between models (X^2 test, $\alpha = 0.05$).

2.3 Results

2.3.1 Exposure history experiment

Grooming - During the secondary exposure, the presence of mites was a significant predictor of grooming frequency ($X^2 = 39.83$, $p < 0.001$, Fig. 2.1 A). However, there was no significant effect of the history of exposure on grooming frequency ($X^2 = 1.00$, $p = 0.32$) and there was no interaction between exposure history and presence/absence of mites during the secondary exposure ($X^2 = 1.07$, $p = 0.30$). Not surprisingly, the presence of mites (during secondary exposure) elicited a strong grooming response. Overall, flies in arenas with mites groomed twice (average across naïve and experienced flies: 13.58 ± 5.71 S.D.) as often as flies in arenas without mites (mean = 7.67 ± 4.16 S.D.).

General movement - The presence of mites during the secondary exposure had a strong effect on frequency of movement ($X^2 = 13.44$, $p < 0.001$, Fig. 2.1 B). Neither the effect of history ($X^2 = 0.25$, $p = 0.62$) nor the interaction between primary and secondary exposure were

significant ($X^2 = 0.67$, $p = 0.41$). In general, flies spent more time moving around the arena in the presence of mites (5.02 ± 4.99 S.D.) than in the absence of mites (2.23 ± 3.27 S.D.). Most likely, the presence of mites resulted in increased movement to avoid contact with mites.

Feeding - Due to a significant interaction between primary and secondary exposure on feeding frequency ($X^2 = 1418.2$, $p < 0.001$), we explored the simple effects using separate generalized linear models with a quasi-Poisson error distribution for naïve and experienced flies. During the secondary exposure, feeding frequency among naïve flies was not significantly affected by mite presence (deviance = 21.22, $p = 0.25$); naïve flies fed a similar amount in the presence (6.97 ± 10.9 S.D.) and in the absence (10.5 ± 14.2 S.D.) of mites. By comparison, mite presence was a significant predictor of feeding frequency among experienced flies (deviance = -71.55, $p = 0.04$, Fig. 2.1 C). Experienced flies spent significantly more time feeding (15.50 ± 13.86 S.D.) upon secondary exposure to mites compared to unexposed flies (8.07 ± 13.02 S.D.).

Resting - There was a significant interaction between primary and secondary mite exposure on resting behaviour ($X^2 = 4.07$, $p = 0.04$, Fig. 2.1 D). Naïve flies did not differ in resting counts in the presence of mites (33.7 ± 12.6 S.D.) and in their absence (39.5 ± 13.8 S.D.) (deviance = -14.12, $p = 0.09$), whereas mite presence was a significant predictor of resting frequency among experienced flies (deviance = -110.38, $p < 0.001$, Fig. 2.1D). Upon a secondary mite exposure, experienced flies spent 20% less time at rest (26.3 ± 11.6 S.D.) than naïve flies (33.7 ± 12.6 S.D.).

2.3.2 Infection history experiment

Grooming - Mite presence during the secondary exposure was a strong predictor of grooming frequency ($X^2 = 17.54$, $p < 0.001$, Fig. 2.2 A). Flies groomed more often in the presence of mites (11.7 ± 5.97 S.D.) than in the absence of mites (7.37 ± 4.67 S.D.), regardless of previous infection history. Neither the effect of infection history ($X^2 = 0.08$, $p = 0.78$) nor the interaction were significant factors ($X^2 = 1.23$, $p = 0.27$).

Ambulatory movement - The presence of mites during the secondary exposure had a significant effect on ambulatory frequency, regardless of infection history ($X^2 = 4.31$, $p = 0.04$, Fig. 2.2 B). In general, flies walked around the arena more often in the presence of mites (3.00 ± 2.79 S.D.) than in the absence of mites (2.07 ± 2.54 S.D.). As predicted, flies increased ambulation to avoid contact with mites. There was neither an effect of infection history ($X^2 = 1.91$, $p = 0.17$) nor a significant interaction between primary and secondary mite exposure ($X^2 = 0.58$, $p = 0.45$).

Tarsal flicking - During secondary exposure, mite presence was a significant predictor of tarsal flicking ($X^2 = 48.51$, $p < 0.001$, Fig 2.2 C), which was expected since tarsal flicking is an important defensive behaviour to prevent mite attachment. Since flies only flicked their tarsi in the presence of mites, we only compared naïve and experienced flies where mites were present in the arena. To test the effects of history on tarsal flicking, history, block, and time of day were analysed as fixed effects in the model (GLM, family = Poisson). There was no effect of infection history (deviance = -0.95, $p = 0.31$). In other words, there was no difference in tarsal flicking frequency between naïve (0.53 ± 0.73 S.D.) and previously infected flies (0.73 ± 0.87 S.D.). Block (deviance = -2.45, $p = 0.63$) and time of day (deviance = -8.45, $p = 0.063$) were not significant factors in tarsal flicking frequency.

Short bursts of flight - Mite presence in the arena was a significant predictor of the frequency of short bursts of flight ($X^2 = 50.71$, $p < 0.001$, Fig. 2.2 D). Flies deployed these short bursts of flight 30-times more frequently in the presence of mites (1.00 ± 1.29 S.D.) than in the absence of mites (0.033 ± 0.18 S.D.). We found no effect of history of infection ($X^2 = 2.03$, $p = 0.16$). The magnitude of increase in bursts of flight was 160% higher among previously infected flies compared to naïve flies. The interaction between primary infection and secondary exposure was not significant ($X^2 = 0.10$, $p = 0.76$).

Feeding - There was no effect of primary infection ($X^2 = 0.24$, $p = 0.62$) or secondary mite exposure ($X^2 = 0.05$, $p = 0.83$) on the feeding frequency (Fig. 2.2 E). In other words, neither infection history nor the presence of mites during secondary exposure influenced the frequency of feeding. The interaction between infection history and mite exposure (during secondary exposure) was also not significant ($X^2 = 1.51$, $p = 0.22$). Time spent feeding was comparable across control and treatment groups.

Resting - The presence of mites ($X^2 = 6.92$, $p = 0.009$, Fig. 2.2 F) was a significant predictor of resting frequency during the secondary exposure. Flies rested less in the presence of mites (27.5 ± 12.1 S.D.) than in the absence of mites (34.0 ± 13.0 S.D.). History of infection did not affect resting frequency significantly ($X^2 = 0.50$, $p = 0.48$) nor was there an interaction between previous infection and secondary mite exposure ($X^2 = 1.13$, $p = 0.29$). This suggests that the frequency of resting is only affected by current mite exposure.

2.4 Discussion

This study aimed to investigate the non-consumptive effects of parasitism in a fly-mite system. We explored whether flies responded to the presence of mites through non-consumptive effects and whether exposure or infection history modulated the expression and magnitude of those NCEs. In both experiments, we found that flies responded to the mere presence of mites, i.e., NCEs of parasites were manifested through changes in host behaviour. As predicted, flies increased grooming and movements in general (ambulation, bursts of flight, tarsal flicking) in the presence of mites to reduce the risk of infection. Increased grooming also promotes the upkeep of sensory organs and the cuticle, and increases pathogen and parasite detection (Zhukovskaya et al., 2013). Flies most likely increased locomotor activity as a form of parasite defense, allowing them to evade the mites and increased locomotion translates into dispersal (Brophy and Luong, 2022). Indeed, adverse habitat conditions such as deteriorating environmental conditions and high mite densities increase the propensity to disperse (Johnston and Heed, 1976). Exposure history (i.e., primary exposure) was not a predictor of grooming and moving frequency. In other words, previous parasite experience did not affect these short-term avoidance responses in subsequent exposures. Given that parasite avoidance behaviours are energetically costly (Luong et al., 2017), the magnitude of the response to mites should correspond to current levels of infection risk and not necessarily past indicators of risk.

Previously exposed (experienced) flies fed twice as much in the presence of mites than flies in mite-free arenas, whereas naïve flies did not show a significant difference in feeding during the secondary exposure. These results are consistent with the hypothesis that organisms learn to avoid parasite cues and subsequently show a stronger response to parasites upon secondary exposure. A previous study found that flies exposed to mite cues exhibit increased metabolic rates, and this additional energy expenditure may result in compensatory feeding (Luong et al., 2017), which would explain the increased feeding among previously exposed flies in our study. Future studies should measure whether history of exposure to exacerbates the impact on fly resting metabolic rate in subsequent exposures to mites. As the probability of encountering a mite increases, flies need to increase their vigilance and defense, resulting in heightened metabolic rates, thus reducing the energy available for somatic maintenance and reproduction. Another possible explanation is that increased feeding may help flies invest in future reproduction. In *D. nigrospiracula*, parasitized male flies increased their reproductive effort

(Polak and Starmer, 1998). Similarly, terminal reproductive investment may play a role; for example, *D. melanogaster* exposed to dead conspecifics increased investment in offspring size (Corbel and Carazo, 2022). Female flies may increase feeding to allocate resources towards future reproductive success following repeat risk of mite infection. The interaction between primary and secondary exposure may also be linked to the ephemeral nature of the cactus rot. In early stages of necroses the mites tend to be free-living, but as the rot dries and food become limited, mites are more likely to parasitize flies as an alternative resource and mode of dispersal (Polak and Markow, 1995). These very conditions may also favour dispersal among flies to escape parasite pressures and locate fresh rots (Brophy and Luong, 2022); an increase in feeding may represent a way to prepare for imminent dispersal following repeated exposures (Edelsparre et al., 2014). These hypotheses are not mutually exclusive.

Experiment 1 also revealed a trade-off between feeding and resting among flies previously exposed to caged mites. Not surprisingly, the increase in feeding among experienced flies resulted in less time spent resting. This trade-off is important because resting (and sleeping) is linked to several *Drosophila* fitness components, including immune function, learning, and oxidative stress (Bryant et al., 2004; Seugnet et al., 2011; Hill et al., 2018). Long periods of immobility are also crucial for predator avoidance, especially for visual hunters (Evans and Schmidt, 1990) and maintenance of basic metabolic functions (Weibel, 2002). Naïve flies also rested less in the presence of mites than in their absence, though that difference was not statistically significant. Because of the interaction between primary and secondary exposure, we did not analyze the main effect of resting. As such, the smaller sample size may account for the lack of statistical power. By comparison, we did detect a significant main effect of mites on resting in experiment 2 (see below), but history of infection and the interaction between primary infection and secondary exposure were not significant.

In the second experiment, we performed behavioural assays to investigate the impacts of primary ectoparasite *infection* and secondary exposure on host behaviour. We hypothesized that primary infection by mites would further increase the expression and magnitude of NCEs during a secondary exposure. We categorized different types of movement to tease apart ambulation as well as defensive behaviours (burst of flight and tarsal flicking). Our results show flies spent more time engaged in walking, grooming, tarsal flicking, and short bursts of flight in the presence of mites. Even though these avoidance and defensive behaviours increased in the

presence of mites as expected, infection history did not have a significant effect on host behaviour during secondary exposure. Flies spent less time resting in the presence of mites regardless of infection history, apparently due to a higher proportion of time spent on other activities. When flies groom, jump, flick their tarsi, or walk, muscle activity increases at the expense of basic metabolic functions (Weibel, 2002). These reflex movements and hyperactivity are adaptive since it decreases the chance of mites attaching (Polak, 2003). Time spent feeding was comparable for naïve and previously infected flies and the presence of mites did not impact this response. We initially hypothesized that the infection history would further strengthen the NCEs during a secondary exposure to parasites. This hypothesis was not supported by our results; none of the behaviours measured were affected by infection history. By comparison the 5-day period of exposure without infection had a strong impact on fly behaviour during the secondary exposure to mites, specifically on feeding and resting. Perhaps a longer or more intense infection period may be required to affect future fly behaviour (e.g., via learning). We chose flies with one to two mites as this is the most common level of infection in the nature (Polak and Markow, 1995; Polak, 1996; Brophy and Luong, 2021). However, if we had increased the intensity of mite infection per fly, any changes in behaviour could be due to the physiological costs of infection and not exclusively due to the "memory" or experience of having been previously infected. Using caged mites in the first experiment allowed for a longer period of exposure to parasite cues, resulting in lasting non-consumptive effects that impacted feeding and resting behaviour in subsequent exposures to mites.

Our findings show that hosts respond to parasite exposure, even without consumption (i.e., infection), through changes in behaviour such as grooming and parasite avoidance. These strategies for avoiding infection are costly and in some cases trade-off with time available for resting. Understanding differences in behaviour arising from parasite exposure and infection is crucial for understanding the ecology of fear and of disgust. Through indirect cues and NCEs, parasites can exert pressure on host populations. Moreover, our results suggest that the non-consumptive effects of past parasite exposure may be stronger and longer lasting than the NCEs of infection history. Parasites not only modify current host behaviour, but have the potential to affect future behaviours, even without active infection.

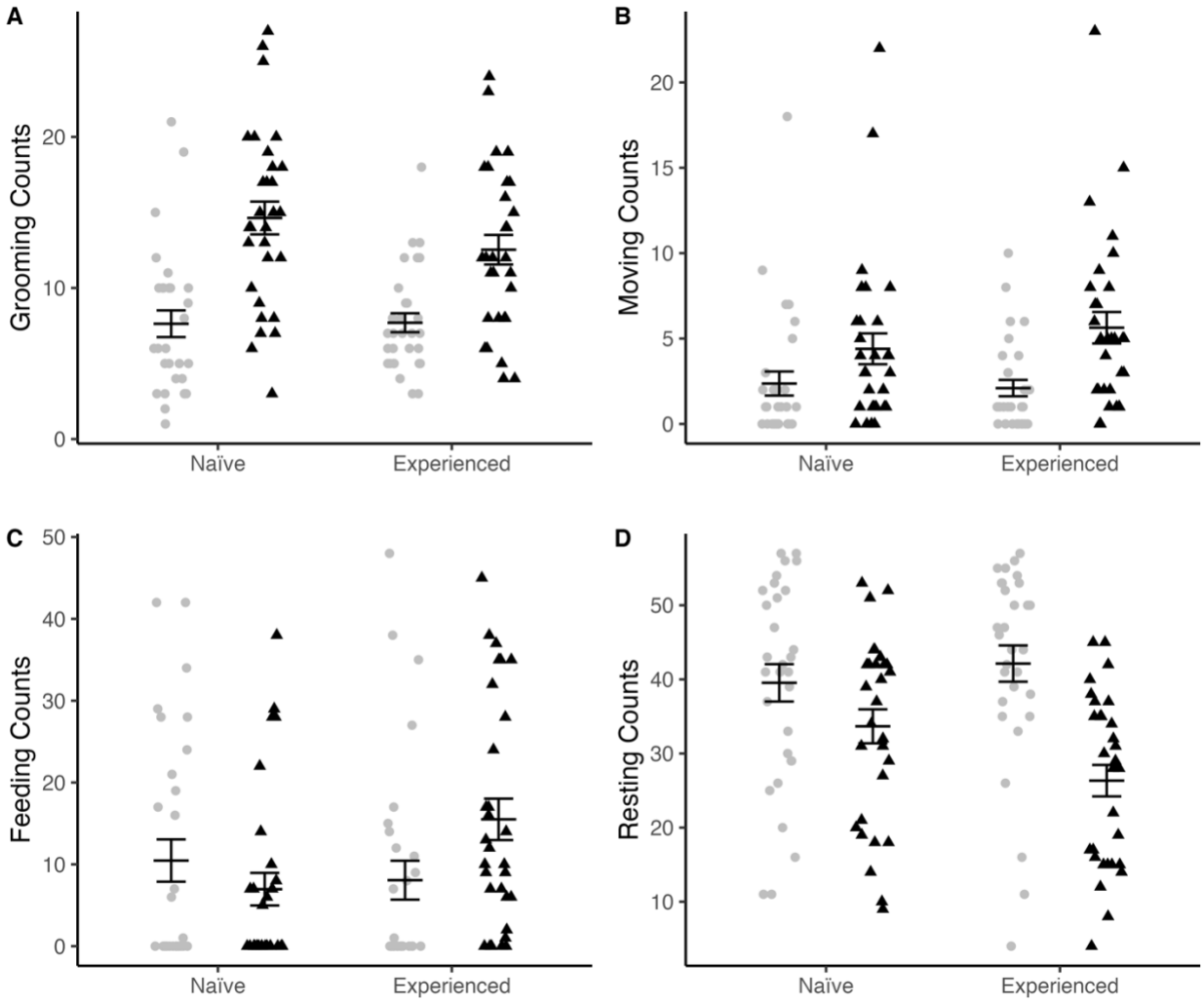

Figure 2.1. Behavioural responses of naïve and previously exposed flies in the presence or absence of mites. (A) Grooming, (B) moving, (C) feeding, and (D) resting behaviour of naïve flies (no history of mite exposure) and flies previously exposed to 5 female mites for 5 days (i.e., experienced flies). Both groups of flies were individually assayed in a petri dish with 7 free-roaming female mites (black triangles) or no mites at all (grey circles), n = 30 flies for each treatment combination. We measured the presence of a given behaviour through scans at every minute point for an hour. Error bars represent standard error, midline represents mean.

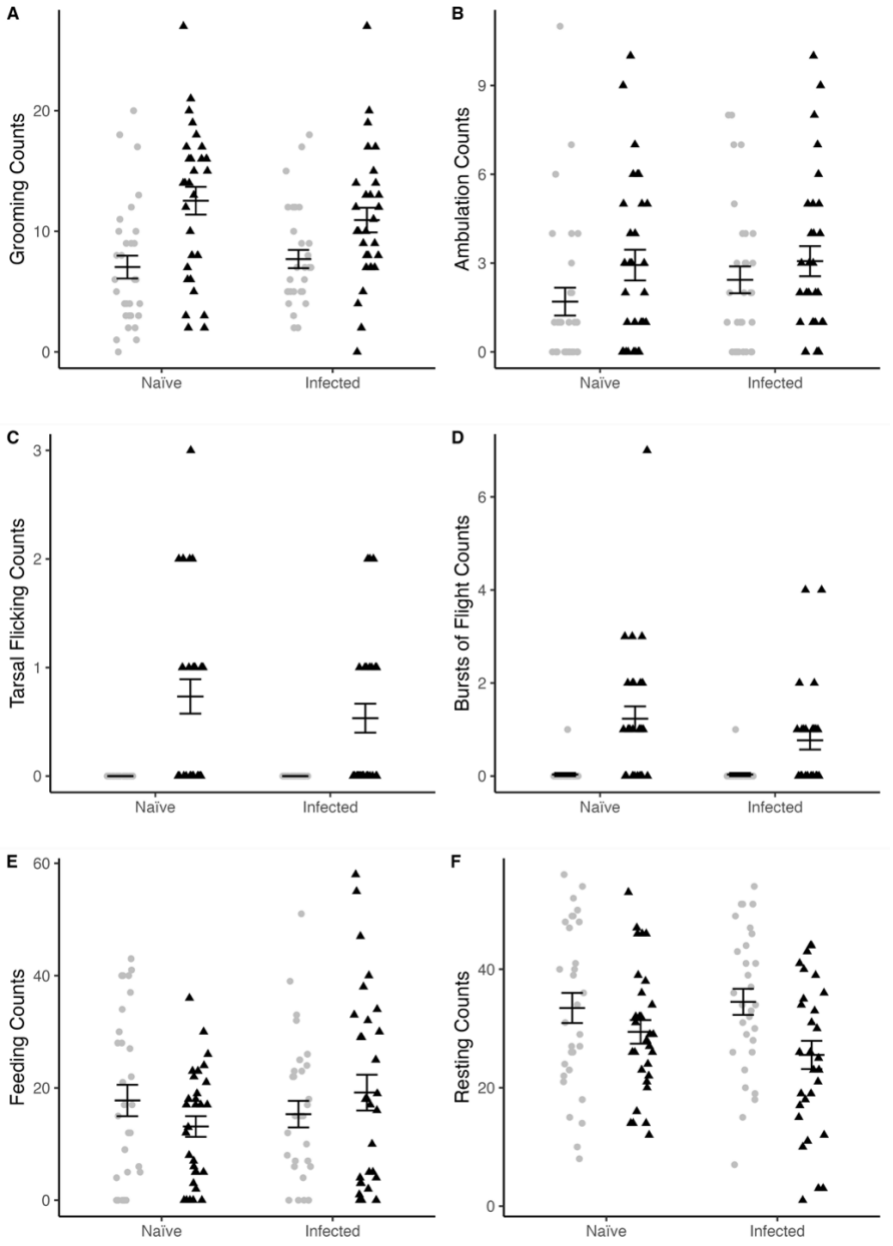

Figure 2.2. Behavioural responses of naïve and previously infected flies in the presence or absence of mites. (A) Grooming, (B) ambulatory, (C) tarsal flicking, (D) burst of flight, (E) feeding, and (F) resting behaviour of naïve flies (no history of mite exposure or infection) and flies previously infected by 1-2 mites for 1 hour. Both groups of flies were then individually assayed in a petri dish with 7 free-roaming female mites (black triangles) or no mites at all (grey circles), n = 30 flies for each treatment combination. We recorded the presence of a given behaviour through scans at every minute point for an hour. Error bars represent standard error, midline represents mean.

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Chapter 3: Carry-over effects on adult phenotype of mite exposure on fly larvae

3.1 Introduction

Predators can reduce prey population by consumption, but the mere presence of predators alone induces stress in organisms, potentially leading to population level consequences (Buck et al., 2018; Clinchy et al., 2013; Peacor and Werner, 2008; Raffel et al., 2008; Rohr et al., 2008). These 'non-consumptive effects' (NCEs) include changes in morphology, physiology, and behaviour, which may result in energetic costs and reduced survival and reproduction (Creel and Christianson, 2008; Hawlena and Schmitz, 2010a, 2010b; Kollross et al., 2023; Nelson et al., 2004; Peacor and Werner, 2008; Preisser et al., 2005; Preisser and Bolnick, 2008). Potential prey may reduce activity, or adapt the timing and location of foraging, as well as shift diet types to reduce predation risk, at the risk of their development and growth (Hawlena and Pérez-Mellado, 2009; Hawlena and Schmitz, 2010a, 2010b; Lima, 1998). Stress also affects metabolic processes, resulting in less efficient conversion of food into biomass (Stoks, 2001). The threat of predation can be chronic and long lasting, therefore the "ecology of fear" may have knock-on effects beyond direct predation alone (Clinchy et al., 2013; Nelson et al., 2004; Pangle et al., 2007).

A growing number of studies show that parasites, like predators, also exert non-consumptive effects (NCEs) on potential hosts (Horn and Luong, 2019, 2018; Raffel et al., 2008; Rohr et al., 2008; Zhukovskaya et al., 2013), and organisms avoid parasite and predator cues to the same extent (Rohr et al., 2008). Since parasite exposure can be chronic, organisms may show stronger cumulative responses to parasites than to predators (Buck et al., 2018; Rohr et al., 2008). The presence of parasites can induce parasite-avoidance behaviours, which are time and energy intensive but are necessary to avoid infection (Buck et al., 2018; Daversa et al., 2021a; Horn and Luong, 2019; Sears et al., 2013; Zhukovskaya et al., 2013). Behavioural changes in the presence of parasites include avoidance of parasite cues (Rohr et al., 2008), reduced activity (Selbach et al., 2022), reduced feeding (Barber et al., 2000; Behringer et al., 2018; Selbach et al., 2022), avoidance of infected conspecifics, habitat avoidance and habitat switching (Daversa et al., 2018; Fritzsche and Allan, 2012; Hutchings et al., 2007, 2002; Rohr et al., 2008). These fear responses to parasites, even without direct contact come at a cost, such as reduced fecundity and longevity (Horn and Luong, 2018).

Predators can exert NCEs on multiple life-history stages of their prey, even on developmental stages that are not typically consumed by the predator (e.g., larval stages of invertebrates) (Ellrich et al., 2016). Short-term exposure of larval *D. melanogaster* to the predator *Phidippus apacheanus* resulted in accelerated development, faster climbing speeds, and increased adult survival in the presence of the predator, at the expense of lower adult body mass (Krams et al., 2016). Predatory cues alone during the larval stage can reduce developmental stability and survivorship to the adult stage, and lead to accelerated emergence, resulting in reduced adult body size, and fewer and smaller offspring (Elliott et al., 2016; Ower and Juliano, 2019; Peckarsky et al., 1993; Stoks, 2001).

There is a scarcity of research on NCEs of parasites on non-susceptible stages, and even less evidence on whether those NCEs have any carry-over effects on adult phenotype. Larvae can detect natural enemies: *Drosophila* larvae show olfactory learning and can avoid parasitoids by detecting their semiochemicals (Ebrahim et al., 2015; Scherer et al., 2003). Responding to those cues and avoiding the parasite would increase survivorship to adult stages. Exposure to a parasite in the larval stages of the flour beetle *Tribolium castaneum* increased resistance, and that change lasted through development (Critchlow et al., 2019). Early exposure to parasites may lead to more resources being allocated toward resistance and parasite avoidance later in life as adults. Consequently, early-stage stress can increase metabolic needs and change body elemental composition (e.g., to maximise escape speeds) and survival (Krams et al., 2016; Thomas and Rudolf, 2010). My goal is to investigate how parasite exposure alone during a non-susceptible sexually immature stage, affects adult fecundity and longevity.

A Sonoran Desert (Arizona, USA) host-parasite association is particularly conducive to investigate the ecology of fear, the fly *Drosophila nigrospiracula* (Diptera: Drosophilidae) reproduces and feeds on necrotic saguaro cactus (*Carnegiea gigantea*) (Danielson et al., 1994; Fellows and Heed, 1972; Markow, 1988) and is infected by the ectoparasitic mite *Macrocheles subbadius* (Mesostigmata: Macrochelidae) (Polak and Markow, 1995). Only female mites are infective and feed exclusively on adult flies (Polak and Markow, 1995; Polak 1993). Since the egg, larval, pupal, and adult stages of *D. nigrospiracula* can be found on the necrotic tissue of the cactus, every life stage is potentially in contact with mites. Adults court and mate on the external surface of necrotic saguaro cactus, females oviposit on newly decaying cactus tissue where larvae undergo development (Markow, 1988). Female flies avoid oviposition in mite-laden areas

(Mierzejewski et al., 2019) and *D. nigrospiracula* larvae preferred pupating on mite-free substrate and had lower pupation success in the presence of mite cues (Horn et al., 2023b). Even though the larval stage is not susceptible to infection, mites exert NCEs on fly larvae survival (Horn et al., 2023b).

We investigated the carry-over effects of mite exposure by identifying differences in adult phenotype brought on by exposure to parasites during the larval stage of development. Specifically, we measured the effects of exposing larvae to parasite cues on adult body mass, fecundity, and longevity. Larval growth and size determine adult body size, and female body size is a significant predictor of fecundity (Lefranc and Bundgaard, 2000). High parasitism risk increases the propensity to disperse (Brophy and Luong, 2021b). If the larval environment has a high parasite density, larvae may accelerate development to disperse to a less mite-laden environment, and this may in turn decreases body size (Ower and Juliano, 2019). Since adult exposure to mites decreases fitness (longevity and fecundity) (Horn and Luong, 2018), larval exposure may result in the same responses. Therefore, we hypothesize that larvae exposed to parasite cues will experience NCEs that carry over into the adult stage and manifest as decreased body condition and fitness. We predict that exposed larvae will have decreased adult body sizes, adult survival, and fecundity.

3.2 Materials and methods

3.2.1 Experimental animals

Drosophila nigrospiracula (Diptera: Drosophilidae) were collected from necrotic saguaro cacti (*Carnegiea gigantea*) in the Sonoran Desert (Arizona, USA) and used to establish laboratory cultures. Flies were reared in 200 mL bottles in media with instant mashed potato flakes, *Drosophila* medium (Formula 4–24 Instant *Drosophila* Medium, Carolina Biological Supply Company, Burlington, NC, USA), nutritional yeast, and 6 grams of autoclaved necrotic saguaro cactus. All fly cultures and experimental flies and larvae were maintained in an incubator (Percival Scientific, Perry, IA, USA) at 24°C and 70% relative humidity (RH) with a 12L:12D cycle. *Macrocheles subbadius* (Acari: Macrochelidae) Berlese were collected from infected flies in the Sonoran Desert and used to establish laboratory cultures. Mites were reared in a 2.5:1 mix of wheat bran and aspen wood shavings (*Populus tremuloides*), with free-living

nematodes and nutritional yeast. Mite cultures were kept separate from fly cultures in a separate incubator (Percival Scientific, Perry, IA, USA) at 25°C and 70% RH on a 12:12 L:D light cycle. Female mites were extracted for experiments using a Berlese funnel.

3.2.2 Larval exposure

In order to obtain a large number of fly larvae for the exposure experiment, larvae were removed with a paintbrush from 6 culture bottles and transferred to a 50 mL specimen cup of 20% sucrose. The sucrose flotation allowed the separation of second and third instar larvae, which tend to float to the solution's surface (first instar larvae typically sink to the bottom after 20 minutes). Larval instar was identified according to Bainbridge and Bownes (1981). A total of 50 second instar (L2) larvae were transferred into a single vial, creating five control and five treatment vials. These larval vials served mainly to concurrently expose a large larval population to mites, generating sufficient numbers of viable adult females to assess the downstream effects on fecundity and longevity. Vials (100 mm in length by 20 mm in diameter) contained fresh media (0.9 g instant mashed potato flakes, 0.25 g *Drosophila* medium, 1 g autoclaved necrotic saguaro cactus, and 5 mL of distilled water). Treatment vials contained 5 female mites that were enclosed in a mite cage (2mL microcentrifuge tube, 40 mm in length by 10 mm in diameter). Both ends of the tube were cropped and sealed with mesh (80 μm pore size) to allow airflow and detection of the mites without direct interaction with the larvae. Control vials had an empty mite cage. The mite cages were suspended 2.5 cm from the top of the vial using cotton twine rope (50 mm in length by 1.58 mm in diameter) to prevent obstruction of the mite cage by the substrate and to standardize the height at which mite cues were diffusing. Once the larvae reach the third instar (~3 days), pupation sites were added to improve pupation success. Developing larvae and pupae were exposed to mites for a total of seven days; mites in the treatment cages were replaced midway through the trial to ensure viability.

3.2.3 Fecundity and longevity

Upon emergence, adult flies were counted and weighed (±0.005 mg, Mettler Toledo XP105, Columbus, OH). The flies were then sorted by sex and treatment groups and housed for a week in agar vials to monitor longevity and fecundity (see below). Seven days post-emergence, a single female fly from the agar vials was re-weighed and moved into a mating vial with two 7-

day old unmated males (from the stock culture) and fresh cactus media. This procedure was repeated for 25 of the flies from the control vials and 25 flies from the treatment vials (5 female flies from each exposure vial). Flies were moved into new vials every 3 or 4 days to reduce larval competition. Males were replaced once a week with 14 day-old males to reduce the effects of age on male courtship and to minimize sperm limitation (Polak and Starmer, 1998). Longevity of the females was recorded with every vial change (every 3-4 days). Vials were monitored every few days for emergence and adult flies were removed as they emerged; offspring were counted and sexed.

3.2.4 Statistical analyses

All analyses were performed using the R Statistical Program (R Core Team 2022). Mixed effect models (lmer function, lme4 package) were used to test whether larval mite exposure and fly sex affected fly emergence weight, with larval exposure vial as a random effect. Emergence success was not analysed since only five replicated vials were initially set up for each exposure condition. Offspring count was analysed with negative binomial generalized linear mixed models where the independent variables included mite exposure treatment, maternal weight, maternal lifespan, and random variables included the maternal date of birth (block) and the fly identifier (GLMM, lme4 package, MASS package). Survivorship was analysed with the Kaplan-Meier proportional hazard regression model (survfit function, Survival Package). The Survdiff function (R, Survival Package) was used to compare survivorship curves between control and exposed flies. Net fecundity of females (L_xM_x: average per vial offspring production, weighted by the probability of surviving to that age class) was log-transformed, and a generalized linear model (family = Gaussian) was used to test whether larval mite exposure and fly age were predictors of net fecundity. There was a total of 12 vial changes (every 3-4 days), so each serial transfer approximated a unit of time (3-4 days); this measure of longevity was used for the life table and net fecundity calculations. Stepwise model selection was performed on the models for emergence weight, offspring count, longevity, and net fecundity. Factors were removed from the models by least significance, and models were compared using the ANOVA function (X^2, $\alpha = 0.05$), and the change in deviance or the X^2 value, and corresponding P-value were reported.

3.3 Results:

3.3.1 Adult weight at eclosion

There was no difference in larval emergence rate between control (24.8 emerged adults ± 2.28) (mean ± SD) and exposed larvae (24.0 emerged adults ± 3.39) (mean ± SD). Control (unexposed to mites as larvae) females weighed on average 2.15 ± 0.40 mg (mean ± SD), and control males were on average 1.79 ± 0.27 mg (mean ± SD). Adult female flies that were exposed to mites as larvae were on average 1.97 ± 0.34 mg and exposed male flies were 1.69 ± 0.27 mg. The effect of treatment on body weight was not significant (X^2= 1.80, p = 0.18). As expected, sex was a significant predictor of emergence weight (X^2= 78.32, p < 0.001), where females overall (2.07 mg ± 0.382 S.D.) were 19.0% heavier than males (1.74 mg ± 0.273 S.D.). Neither the date of birth (block) (X^2= 0.87, p = 0.65) nor the interaction between sex and treatment were significant predictors of weight (X^2= 0.27 p = 0.60).

3.3.2 Offspring count

We examined the effects of mite exposure (during the larval stage) on adult fecundity (in terms of total offspring count). Mite exposure was not a significant predictor (X^2 = 0.06, p = 0.80) of offspring count: control flies produced on average 354.4 ± 43.2 (mean ± SD) offspring and exposed flies produced 329.9 ± 41.6 (mean ± SD) offspring. Lifespan was a significant predictor of offspring count (X^2 = 40.521, p < 0.001). As the lifespan of flies increased, their reproductive output increased correspondingly, regardless of previous mite exposure. There was also a significant positive effect of maternal weight (X^2 = 7.71, p = 0.006) on offspring count.

3.3.3 Longevity

Mite exposure was not a significant predictor of lifespan: exposed flies had a 7% increase in longevity compared to control groups, though that difference was not significant (X^2 = 0.04, p = 0.84). Control flies survived 26.6 ± 8.66 (mean ± SD) days and exposed flies survived 28.5 ± 8.84 (mean ± SD) days post-emergence. The survival curves of control and exposed flies were not significantly different (Survdiff, X^2 = 0.5, p = 0.5) (Fig. 3.1). Mite presence did not affect longevity and mite-exposed flies did not appear to experience an early die off.

3.3.4 Net fecundity

To account for the effects of longevity on offspring count, we then calculated net fecundity (L_xM_x). Maternal age class was a significant predictor of net fecundity (deviance = -64.1, $p < 0.05$): net fecundity decreased with age as the probability of surviving to that age decreased. Exposure treatment was not a significant predictor of net fecundity (deviance = -0.160, $p = 0.74$) (Fig. 3.2): there was no difference in net fecundity between exposed and control females. Net fecundity among control flies decreased with age and peaked at 10-12 days post-eclosion ($\log(L_xM_x) = 4.41$). Net fecundity among females exposed to mites as larvae peaked later than control flies at 16-19 days post-eclosion ($\log(L_xM_x) = 4.26$). However, there was no significant difference in the age at which flies peaked in fecundity since the interaction between treatment and age on net fecundity was not significant (deviance = -0.102, $p = 0.80$).

3.4 Discussion

This study tested the hypothesis that the non-consumptive effects of mites on larvae persist through fly development. We investigated whether flies respond to the presence of mites in their larval stage, and whether these responses persist through development with changes in adult body weight, reproductive success, and longevity. Contrary to our prediction, we did not observe an effect of mite presence on larval development since there was no difference in adult emergence weight between control and exposed flies. Only sex was found to be a predictor of emergence weight, which is expected since female *Drosophila* are generally larger than males (Blanckenhorn et al., 2007; Nunney, 2007). Adult body size may be dictated by factors such as egg size, initial larval size, growth rate, and development time (Meister et al., 2018). Consequently, adult size may already be determined at the second instar. Adult female flies did not suffer reduced fecundity nor reduced lifespan when exposed to mites during the larval stage. We found that maternal weight and lifespan impacted offspring count, which is as expected since increased insect body size is linked with increased fecundity (Honěk, 1993; Meister et al., 2018) and survival (Beukeboom, 2018). The lack of difference in body size may then explain the absence of impact from larval exposure on offspring count, net fecundity, and survival.

Our study did not find any quantifiable NCEs: this could be due to the quality of the larval environment. In a previous study, larvae exposed to mites had lower pupation rates and preferentially pupated in mite-free zones, but there were only marginal effects of treatment on

adult mass (Horn et al., 2023b). The exposure vials we used were larger with more headspace than the Petri dish arenas used in the study by Horn and colleagues (2023), and the relatively larger vials in our study may have resulted in more diffuse mite cues. Mite cages were suspended above the media, which prevented the media from clogging the cages, but also increased the average distance between larvae and parasite cues. The experimental set up did not offer larvae a choice between mite-free and mite-laden environments for pupation. Habitat switching and parasite avoidance (Daversa et al., 2018; Horn et al., 2023b) are effective mechanisms to mitigate infection risk. Fly larvae can actively evade predators, parasites, and parasitoids (Ebrahim et al., 2015; Horn et al., 2023b; Krams et al., 2016). Our set up precluded fly larvae from evading mite cues, therefore eliminating the main defensive behaviour larvae typically exhibit in response to mites (Horn et al., 2023b). Additionally, larvae in nature burrow into the cactus media, thus decreasing their exposure to parasite cues while foraging in a highly nutritious, mite-free refuge (Danielson et al., 1994; Fellows and Heed, 1972). Therefore, the media in the vials was about 1.5 centimeters deep, and it may represent high quality refuge that mitigated the fear-related effects of mite exposure. Changes in future study design would include increasing the concentration of mite cues to increase the strength of the fear response of larvae, which could then have larger downstream effects through development into the adult stage. Another way to modify the larvae to mite ratio would be to decrease larval density. In *D. melanogaster*, at low densities of adults (less than 20 individuals per vial), there was a greater impact of predation cues on fecundity and offspring mass (Elliott et al., 2017, 2016). Reducing larval density in future experiments could allow the detection of NCEs.

Exposure to predation cues can have different effects on potential prey depending on nutrient level. In *D. nigrospiracula*, M. subbadius infected females had higher fecundity with yeast-supplemented diets compared to uninfected females with yeast-free diets (Polak, 1996). Certain trade-offs would not be possible in low nutrient environments: organisms may not trade-off foraging with parasite or predator avoidance. Ower & Juliano (2019) found that at high nutrient levels, females emerged sooner (than in low nutrient conditions) in the presence of predator cues and but did not show decreased body size. The authors argue that nutrition may reduce the overall effects of predatory cues on survival (Ower & Juliano, 2019). Under conditions of nutritional stress, there may be larger differences in emergence weight; for example if the risk of parasitism affects the amount of reserves accumulated (Ower and Juliano, 2019),

that could lead to the changes in longevity and reproduction. Future experiments could test whether larvae exposed to parasites accelerate their development. Increasing the rate of development would also allow larvae to escape the threat of parasitism (Ower and Juliano, 2019). Future studies could investigate whether nutrient constraints might interact with parasite presence and affect development time and adult size. In our study, reducing the amount of cactus media would address the problem of the media serving as a mite-free refuge, and would impede on larval reserve accumulation, which would ultimately emphasize the effects of mite cues during larval development.

We only used female flies in our experiment since parasite-induced risk is higher for females than males as mites preferentially infect female flies. However, there is stronger sexual selection in males: sexual selection in male *Drosophila* includes competition for access to mates and for fertilization post-copulation (Davies et al., 2023; Morimoto and Wigby, 2016). In *D. nigrospiracula*, infection by *M. subbadius* elevates reproductive effort (Polak and Starmer, 1998) and in mosquitoes, there is evidence that the non-consumptive effects of predation may result in bigger life-history costs in males than in females (Costanzo et al., 2011). Exposure to mites as larvae may increase post-sexual maturation reproductive effort of male flies, thus increasing mating success and lifetime fecundity. This is supported by a simulated fly-mite population model: fly populations experiencing NCEs without consumption had greater population growth compared to unexposed populations, thus suggesting reproductive compensation (Horn et al., 2022). In chapter 2, we found that experienced flies (that had primary mite exposure) increased feeding upon a secondary mite exposure, which could be due to compensatory reproduction. Quantifying and comparing compensatory reproduction in both sexes would allow a more comprehensive study of NCEs.

Alternatively, it is possible that the NCEs of mite exposure on larvae simply do not persist through development. If the adult's environment has no or few mites present, the lack of any carry-over effects could be beneficial to hosts, i.e., if they can disperse to mite-free habitats. Hence, natural heterogeneities in parasite risk could affect the expression and magnitude of NCEs over the lifespan of host. The ecology of fear of parasites therefore hinges on the detection and persistence of parasite cues in the environment, resulting in non-consumptive effects.

Parasites are ubiquitous in the environment, and the probability of encountering a parasite may outweigh that of a predator (Windsor, 1998). Parasites expands the possibilities for ecology

of fear research: coinfections involving multiple parasites, immunisations against endoparasites, and predator-parasite-host interactions present intriguing avenues for exploration. The resulting non-consumptive effects of parasites can alter potential host behaviour and life-history traits, and those changes can cascade into population, community, and even ecosystem-level impacts. NCEs may include changes in trophic interactions, resource, and landscape use, highlighting the importance of host-parasite dynamics. Non-consumptive effects have potentially greater impact over consumption itself, therefore incorporating the NCEs of predators or parasites into interaction networks can provide valuable insight. The ecology of fear has wide reaching implications, with applications in biological pest control (Ingerslew and Finke, 2020), or disease transmission management (Russell et al., 2022) where it can inform effective management strategies for ecologists.

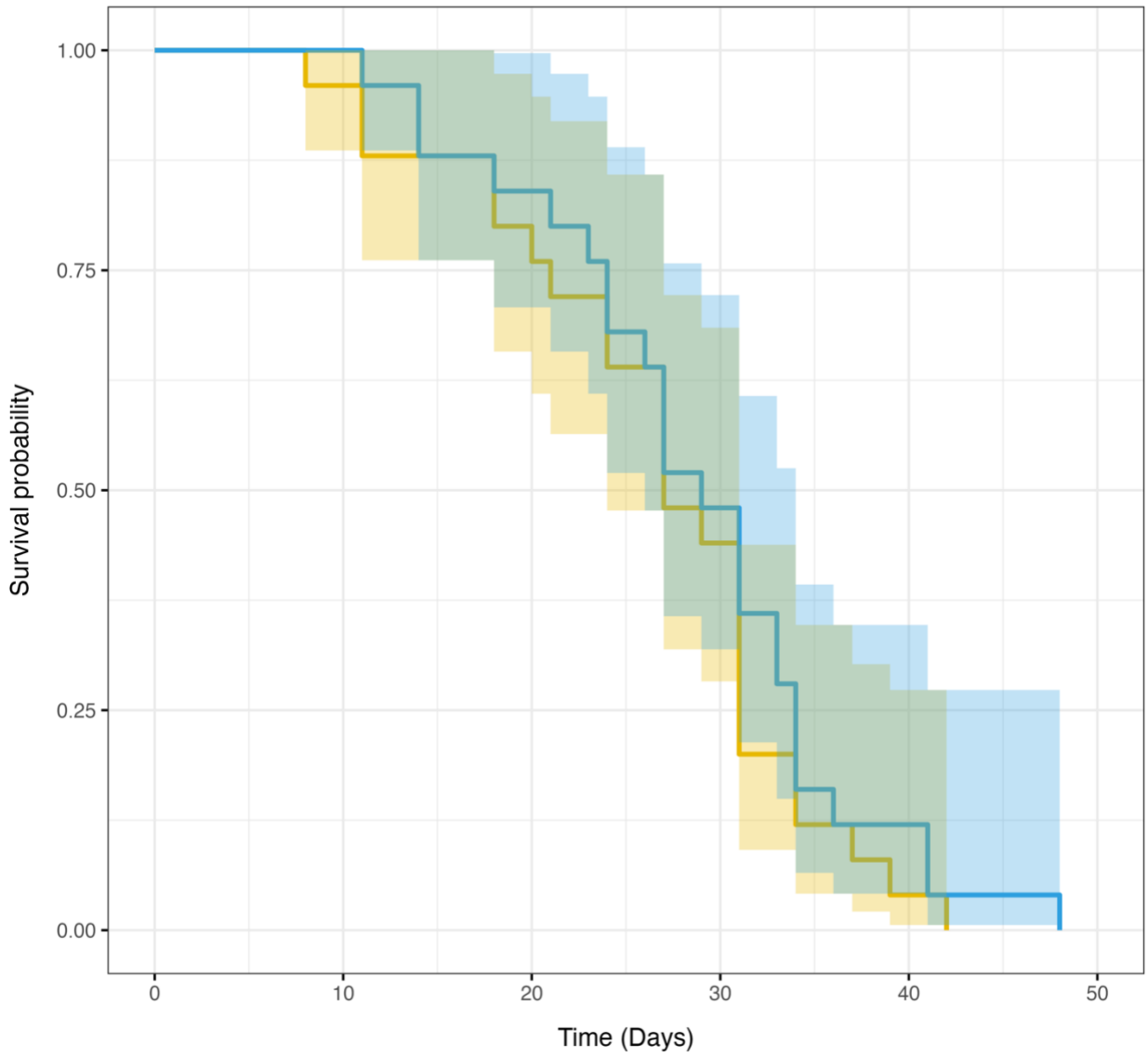

Figure 3.1. Survivorship curves for flies that were either exposed to mites as larvae (n = 25, blue line) or not exposed (n=25, yellow line). The survivorship curves are not significantly different (Survdiff, $X^2 = 0.5$, p = 0.5). Translucent regions represent 95% confidence interval.

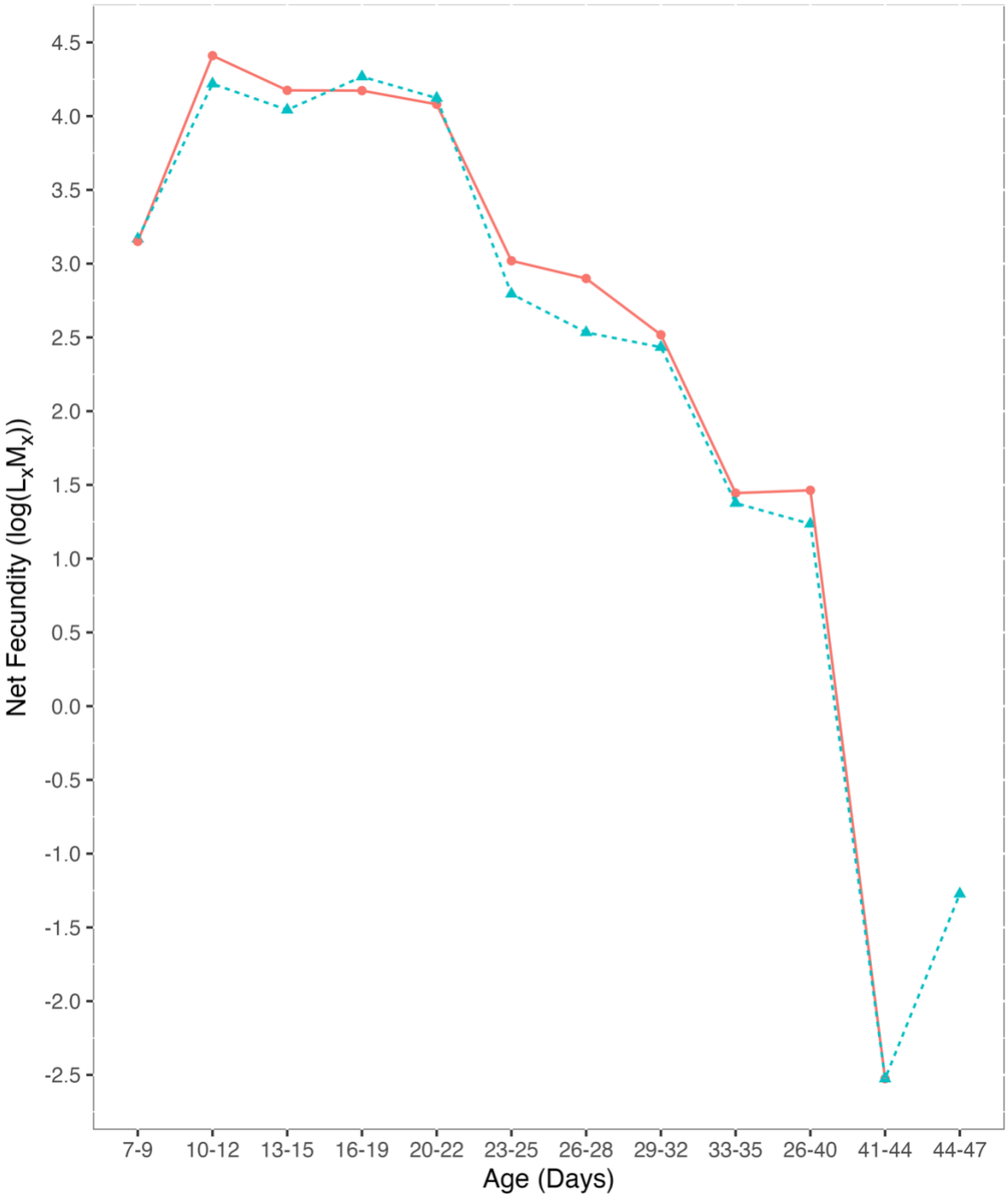

Figure 3.2. Net female fecundity ($\log(L_xM_x)$) across age classes. Control flies (red circles, n = 25) had no history of mite exposure and mite-exposed flies (blue triangles, n = 25) were exposed to 5 female mites as larvae. There was no difference in net fecundity between treatments (deviance = -0.160, p = 0.738).

Chapter 4: Conclusion

4.1 Thesis conclusions

The non-consumptive effects (NCEs) of parasitism have been studied in various systems in the context of ecology of fear, which was a term adopted form the predator-prey literature. More recently, the literature has shifted focus to the "ecology of disgust" whereby potential hosts avoid parasite-laden environments, foods, and conspecifics (Buck et al., 2018; Daversa et al., 2021b, 2018; Doherty and Ruehle, 2020; Gibson and Amoroso, 2022; Hutchings et al., 2007, 2002; Rohr et al., 2008; Weinstein et al., 2018a). While disgust is effective against pathogens entering the host through the oral pathway or other passive routes of transmission, it provides limited protection against parasites that actively search out their hosts, such as ectoparasites (Kupfer and Fessler, 2018). In the *Drosophila nigrospiracula-Macrocheles subbadius* association, the ectoparasitic mites actively seek out flies, therefore avoidance alone in a "landscape of disgust" is not enough to prevent infection (Perez-Leanos et al., 2017; Weinstein et al., 2018a).

My thesis examines the ecology of fear and the NCEs of parasitism in a fly-mite system. In chapter 2, I examined the behavioural changes in adult flies in the presence or absence or mites, and how previous exposure and infection with mites might shape future behaviour. Not surprisingly, parasite presence resulted in elevated avoidance behaviours in flies, including increased grooming, ambulation, tarsal flicking, and exhibited higher frequency of short bursts of flight. These first-line behavioural defenses includes increased ambulation and bursts of flight allow organisms to move away from parasites, and grooming and tarsal flicking decrease ectoparasite attachment success (Benoit et al., 2020; Polak et al., 2023; Zhukovskaya et al., 2013). Behavioural defences can be quite time consuming: various host-parasite systems show evidence that increasing ectoparasite density increased the frequency of grooming at the detriment of resting (Giorgi et al., 2001; Hawlena et al., 2007; Tripet et al., 2002).

I experimentally showed that prior mite exposure, rather than infection affected host behaviour. Chronic parasite exposure, even without infection and with a subsequent recovery period, led to an interaction between exposure history and current mite exposure. Flies exposed to mites 3 days post eclosion increased feeding and decreased resting upon a secondary exposure to mites in the observation arena at 12 days of age, while naïve flies did not show changes in

either feeding or resting frequency regardless of mite presence in the arena. In contrast with our results, aphids decreased feeding in the presence of damsel bugs, even when damsel bug mouthparts were altered to prevent predation (Nelson et al., 2004). One possible explanation for the increased feeding upon a secondary exposure is compensatory feeding for reproduction. A simulated fly-mite population model showed that fly populations experiencing NCEs without consumption had greater population growth compared to unexposed populations, which can be explained by reproductive compensation (Horn et al., 2022). When resources are limited, trade-offs between flight (or dispersal) and reproduction can arise (Marculis et al., 2020; Zera and Denno, 1997). Increased feeding can reduce trade-offs between reproduction and dispersal, and (Zera and Denno, 1997). Therefore, parasite-induced increases in feeding can benefit the host as it provides more energy for parasite defense and reproduction, but any resulting increased host body sizes also benefits the parasite (Bernardo and Singer, 2017; Hechinger et al., 2013; Horn et al., 2023a; Poulin and George-Nascimento, 2007). The interaction between primary and secondary exposure on resting may also signal a trade-off such that increased defense and feeding behaviours interrupt periods of rest. Elevated activity coupled with reduced periods of rest may increase vulnerability to predators and disrupt the maintenance of basic metabolic functions (Bryant et al., 2004; Evans and Schmidt, 1990; Seugnet et al., 2011; Weibel, 2002). In contrast, infection history had no effect on any of the behaviours measured in chapter 2.

Chapter 3 represents the first study to follow the effects of *M. subbadius* exposure on *D. nigrospiracula* larvae through development. I tested whether the NCEs of mite exposure on larvae would carry over into the adult stage. I predicted that stress imposed by mite presence on larvae would result in reduced adult body sizes (through accelerated development), altered reproduction, and decreased survival. These changes may trade off with increased ectoparasite resistance, and that may enhance fitness in the presence of mites. Contrary to our prediction, we did not find any effect of larval exposure on adult body weight, lifespan, and fecundity. Perhaps the mite cues were too diffuse in the exposure arena, or the larvae escaped the parasite cues by burrowing into the media. Alternatively, the NCEs of mite exposure on larvae might not persist into adulthood if the adult environment lacked mites, and the anticipatory parental effects hypotheses require the adult environment to match the larval environment in terms of parasitism risk. We assumed that mite presence would result in suboptimal conditions; however, the nutritious quality of the vials may have resulted in a “silver spoon” effect where a high-quality

diet compensated for any fitness costs of early mite exposure (Pigeon et al., 2019). Food compensation may be an effective mechanism of averting fitness costs in terms of offspring quantity and quality (Tripet et al., 2002; Tripet and Richner, 1997). In less nutritious conditions, compensatory feeding may not be possible, and increasing defensive behaviours may be the sole mechanism to avoid the fitness costs of parasite infection (Cotgreave and Clayton, 1994; Luong et al., 2017).

Prey may exhibit changes in life-history traits in response to predator threat during the course of several generations, even with predator cue removal (Walsh et al., 2015). Therefore, effects of parasite exposure may only manifest after multiple generations. Selection may favour potential hosts that exhibit robust NCEs if these behavioural or morphological changes increase fitness. Even with predator cue removal, potential prey still exhibit NCEs, therefore the lack of parasites in the adult vials could still lead to NCEs if several generations were exposed to parasites (Walsh et al., 2015). After only one generation, changes in body weight, fecundity, and longevity may not be detectable.

In chapter 3, only offspring counts were measured, but studies have shown that maturation rates can be a plastic response to predation that is heritable across generations (Walsh et al., 2015). In an experiment by Walsh and colleagues (2015), distinct strategies adopted by *Daphnia* in response to predator cues resulted in different transgenerational outcomes. Individuals that hastened their maturation and increased offspring production in the presence of predator cues did not exhibit intergenerational responses: subsequent generations did not exhibit earlier maturation. Conversely, *Daphnia* that delayed maturation showed robust transgenerational responses, where their offspring similarly delaying maturation (Walsh et al., 2015).

In our experiment, fly larvae environmental conditions were not similar to adult environment: only larvae had mites in their environment. If parental flies modify traits of future generations to thrive in mite-laden environments ("silver spoon effect"), their progeny may have phenotypes that increases their resistance or avoidance of parasites (Agrawal et al., 1999). We only measured offspring count, but future experiments investigating maternal effects of NCEs should measure offspring resistance to parasites. In *Daphnia*, mothers that were exposed to predator kairomones produced progeny with bigger helmets, which are an energetically costly anti-predatory morphological feature (Agrawal et al., 1999; Riessen, 1984). Two generations

post-predator cue removal, *Daphnia* still exhibited earlier maturation and increases in reproductive output (Walsh et al., 2015).

4.2 Limitations and future directions:

Although we detected changes in frequency of certain behaviours, video monitoring software (such as EthoVision) should be used in future studies to increase the precision of behavioural scans. The speed and direction of fly movement can be tracked as well as the duration of grooming. Video analyses would provide increased resolutions of time budgets compared to scans once a minute for an hour. However, visual observations allowed to measure multiple flies during the same hour thus decreasing any variation in behaviour due to time of day. Videos may also compromise the accuracy of behaviour quantification, particularly for activities such as feeding, where flies may appear immobile while feeding on a food patch, making it challenging to distinguish between feeding and resting behaviours.

Drosophila melanogaster food intake can be directly measured using various methods such as capillary feeders (CAFE) for liquid foods and consumption-excretion assays (Con-Ex) for solid foods (Shell et al., 2018; Wong et al., 2009). The Con-Ex method involves dyeing food and then measuring differences in absorbance through spectrophotometry (Shell et al., 2018). Future studies investigating compensatory feeding could assess food intake to determine whether flies increase bouts of feeding or increase consumption levels. Given my objective to solely measure differences in time allocation across different behaviours, a simpler approach was suitable.

Flies may exhibit habituation with repeated mite exposure without infection and the costs of parasite-defence may not be measurable within an hour-long observation period. Future experiments should extend the duration of fly-mite interactions. To achieve this, flies and mites would be put in a controlled setting, such as a Petri dish, where infection rates are low. The smooth surface and size of the Petri dish hinders the infection success of mites, since mites are more effective in close proximity environments, such as infection jars. This setup would require flies to remain vigilant and continuously fend off potential infections for a few days. Then, any increases in energetically costly behaviour would have more pronounced consequences: changes in fly weight, endurance, and metabolic rates can be measured. Combining the objectives of both

chapters, repeated mite exposure in sexually mature flies could reveal whether flies show compensatory reproduction post-exposure.

Ectoparasites reduces reproductive performance even without infection (Horn and Luong, 2018; Peckarsky et al., 1993). Chapter 2 shows that parasite cue detection is imperative to generate NCEs, and the lack of effect due to parasite exposure in chapter 3 can be resolved by increasing mite cue density, allowing direct contact with mites, and reducing larval proximity to mites. Larvae may be prompted to respond to higher mite densities as it may signal a heightened risk of parasitic infection in the environment. Future studies should consider direct exposure of larvae to free-roaming mites as this allows for both chemical and physical cues (Horn et al., 2023b). Larval exposure experiments that allow mites to infect adult flies post-eclosion would result in selection over several generations for mite resistance: larvae that invest in reproduction and that accelerate emergence may have higher fitness. If we find evidence of phenotypic changes, such as accelerated development or increased endurance, we can observe whether these mite-induced changes are persistent by removing mites from the environment.

4.3 Significance:

This study has contributed robust knowledge to the field of ecology of fear in host-parasite associations. We demonstrated that NCEs can vary over time and space: fly larvae were "rescued" from the NCEs of mite exposure when reaching the adult stage, i.e., there were no lasting effects of mite exposure on adult fecundity and survival. Since mites were not present in the adult environment, varying environmental conditions, such as mite densities, may modulate the effects of fear. We also showed that parasites exert NCEs on susceptible adult flies, and these include changes in time allocation to behaviours such as feeding and resting. Increasing defensive behaviour comes at a cost and can explain the non-consumptive loss of fitness exhibited in multiple host-parasite systems. Even when absent, parasites influenced feeding and resting frequency; therefore, parasites may have broader impacts than expected. Given that parasites represent 75% of all trophic linkages (Dobson et al., 2008; Windsor, 1998) and that parasites likely exert various underreported NCEs, ecologists may be underestimating the cascading effects of fear-induced behavioural responses throughout ecosystems.

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