SEXUAL CONFLICT, SOCIAL NETWORKS, AND POLYANDRY

EXAMINING THE INTERPLAY BETWEEN SEXUAL CONFLICT, SOCIAL NETWORKS, AND POLYANDRY

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LAY ABSTRACT

Sexual conflict occurs when the reproductive interests of males and females are not in alignment with one another. A common form of sexual conflict occurs when males want to mate more often than females, resulting in harassment of females. Such conflict between the sexes over mating is common across the animal kingdom. While there are many evolutionary consequences of sexual conflict, little is known about how sexual conflict influences the social behaviours of animals. For my thesis, I used bed bugs (*Cimex lectularius*) to bridge the gap between sexual conflict and social behaviour. I showed that bed bugs are under intense sexual conflict over mating rates which influences both females' social preferences and their behavioural responses to males. I also found that bed bug females often mate with multiple males, which plays a large role in male mating behaviours and strategies. Finally, using fruit flies (*Drosophila melanogaster*), I show that mating with multiple males can sometimes be highly beneficial to females. The results of my studies have important implications for understanding the evolution of social and sexual behaviours in both sexes.

ABSTRACT

Sexual conflict occurs when the reproductive optima of males and females are at odds with one another. Conflict between the sexes is ubiquitous across the animal kingdom and is expected to influence the social dynamics of group-living animals. Yet, most social behaviour studies do not address the potential ramifications that sexual conflict can have on social interactions. For my thesis, I used bed bugs (*Cimex lectularius*) to bridge the gap between sexual conflict and social behaviour. In Chapter 1, I developed a novel semi-naturalistic arena for tracking bed bugs to uncover how sexual conflict shapes animal social networks. My results show that male and females can be in conflict over the social environment. In Chapter 2, I examined how female sexual history shapes mating interactions using bed bugs. First, I showed that realistically high rates of traumatic insemination relative to lower rates dramatically reduce female fitness. Next, I manipulated female insemination status in a realistic group setting and found that males can exhibit strong mate choice even in a mating system with seemingly little male reproductive investment. Lastly, I tracked avoidance behaviour exhibited by female bed bugs as they received successive inseminations and demonstrated that female bed bugs possess plastic avoidance strategies based on their mating history. In Chapter 3, I examined how social experience shapes sexual interactions in a complex, competitive environment and found that social experience did not improve male or female bed bugs' sexual competence. Finally, in Chapter 4, I extended my work on polyandry to fruit flies (*Drosophila melanogaster*) and showed that realistically high rates of female multiple mating can increase female fitness. In each chapter, I discuss the significance of my findings as they relate to sexual selection and the evolution of social and sexual strategies and behaviours in both sexes.

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DECLARATION OF ACADEMIC ACHIEVEMENT

This dissertation is organized according to McMaster University's approved sandwich thesis format and consists of six chapters. **Chapter 1** introduces the thesis and provides an overview of the subsequent data chapters. **Chapters 2 to 4** are published manuscripts and **Chapter 5** is a submitted manuscript currently in review. **Chapter 6** provides a summary of my main findings and a discussion of future avenues of research.

CHAPTER 1 – Introduction
Author: Janice L. Yan

CHAPTER 2 – The social consequences of sexual conflict in bed bugs: social networks and sexual attraction
Authors: Janice L. Yan and Reuven Dukas
Publication: Animal Behaviour, 192: 109 – 117 (2022).
Comments: J.L.Y. and R.D. conceived the study. J.L.Y. conducted the experiments and analyzed the data. J.Y. and R.D. wrote the manuscript.

CHAPTER 3 – Sexual conflict and sexual networks in bed bugs: the fitness cost of traumatic insemination, female avoidance and male mate choice
Authors: Janice L. Yan, Maggie L. Dobbin, and Reuven Dukas
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CHAPTER 4 – Sexual conflict and social networks in bed bugs: effects of social experience
Authors: Janice L. Yan, Jack R. Rosenbaum, Selena Esteves, Maggie L. Dobbin and Reuven Dukas

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CHAPTER 6 – Discussion
Author: Janice L. Yan

CHAPTER 1 – INTRODUCTION

1.1 General Introduction

When Darwin (1871) laid the foundations of sexual selection theory in *The Descent of Man*, he described in detail the general pattern of males exhibiting a greater eagerness to mate compared to females. While Darwin's natural observations were foundational to the fields of evolutionary biology, sexual selection, and animal behaviour, he was unable to pinpoint an underlying reason for why the sexes differed in their willingness to mate. Recognizing this knowledge gap, Bateman (1948) conducted several experiments examining the relative fertility of male and female fruit flies (*Drosophila melanogaster*) that were housed in groups and therefore naturally varied in their mating history. Bateman's experiments showed greater variance in reproductive success as well as a stronger correlation between number of mates and fertility in males compared to females. While these experiments contained several methodological weaknesses, they still produced significant insights. Most notably, Bateman noted that since males produce large quantities of energetically cheap gametes compared to females who produce fewer but more energetically expensive gametes, male reproductive success is typically constrained by access to females, or more precisely their eggs, while females' reproductive success tends to be constrained by access to resources rather than male gametes. Consequently, optimal mating rates tend to be higher for males than for females. Though initially ignored, Bateman's ideas were later resurrected when Trivers (1972) formulated his theory of relative parental investment which further described the sexes' asymmetric investment in reproduction. Not long after, Parker (1979) formally described the idea of sexual conflict to broadly capture instances where the reproductive interests of males and females are directly at odds with one another.

The seminal works by Trivers (1972) and Parker (1979) marked a paradigm shift from the view that reproduction was generally a cooperative process to instead, a domain of competing interests between the sexes. Since then, decades of research have focused on the co-evolution of sexually antagonistic traits where males evolve traits that are harmful towards females and females co-evolve traits aimed at countering such harms (Chapman,

2006; Lessells, 2006; Perry & Rowe, 2015). Throughout the past few decades, many sexually antagonistic traits like barbed genitalia and coercive behaviours have been thoroughly described in males (Baniel et al., 2017; Baxter et al., 2019; Clutton-Brock & Parker, 1995; Crudgington & Siva-Jothy, 2000b; Dukas et al., 2020; Dukas & Jongsma, 2012; McKinney & Evarts, 1997). Fewer female resistance traits, however, have been well-characterized.

Beyond the evolution of antagonistic traits, reproductive conflict between the sexes is thought to influence other important aspects of biology like aging (Bonduriansky et al., 2008), population structure (Eldakar et al., 2009), speciation (Gavrilets, 2014; Parker, 1998), extinction (Kokko & Brooks, 2003) and social interactions (Darden et al., 2009; Martens & Rehfeldt, 1989; Stanley et al., 2018). Integrating sexual conflict theory into other sub-disciplines of behavioural ecology such as social behaviour can lead to novel insights about the various selective pressures that shape animal behaviour and more importantly, how these different selective pressures interact. Moreover, the recent introduction of social network analysis to the field of animal behaviour creates a unique opportunity to examine social and sexual dynamics from more ecologically relevant settings. Adopted from a branch of mathematics known as graph theory, this set of statistical and graphical techniques allows us to characterize complex patterns of interactions between individuals in a group setting (Croft et al., 2008). As such, the application of social network analysis to animal behaviour has played a crucial role in revealing new insights about disease transmission (Alciatore et al., 2021; Bull et al., 2012; Stroeymeyt et al., 2018), information spread (Aplin et al., 2012; Valentini et al., 2020), resource sharing (Leu et al., 2011; Samuni et al., 2018), and collective decision-making (Kashetsky et al., 2023). By using the connections within a network to represent sexual interactions, social network analysis can also be used to examine mating success (Beck et al., 2021; Tregenza et al., 2019), sperm competition (Fisher et al., 2016; McDonald & Pizzari, 2017), and how patterns of mating within a population shapes sexual selection (Greenway et al., 2021; McDonald & Pizzari, 2016).

Lastly, knowing whether sexual conflict is occurring at all and determining the strength of sexual selection operating on each sex heavily relies on quantifying the economics of mating (ie. the fitness costs and benefits accrued by each sex as they mate with an increasing number of partners). Previous descriptions of conventional sex roles in animals have often emphasized the benefits and relatively low costs of mating for males given their cheap and numerous gametes. As a result, securing a large number of mates has often been seen as the main route towards maximizing fitness for males (Bateman, 1948; Clutton-Brock & Parker, 1992; Emlen & Oring, 1977). However, documentation of sperm and seminal fluid limitation across taxa (Birkhead, 1991; Preston et al., 2001; Reinhardt et al., 2011; Wedell et al., 2002), and polyandry combined with the existence of non-random patterns of sperm usage like cryptic female choice and sperm competition (Parker, 1970; Thornhill & Alcock, 1983), imply that more nuanced and complex mating strategies are likely required in order for males to maximize their fitness. For example, in a species with last male sperm precedence, males may benefit more from defending their existing mates from re-mating with rival males instead of trying to secure as many mates as possible (Harts & Kokko, 2013; Parker & Pizzari, 2010).

Likewise, previous descriptions of conventional sex roles have historically assumed that females have little to gain from mating with several males (Bateman, 1948; Clutton-Brock & Parker, 1992; Emlen & Oring, 1977). However, since the Polyandry Revolution of the 1970's, it has become increasingly clear that females across taxa often mate with multiple males (Parker & Birkhead, 2013; Pizzari & Wedell, 2013; Taylor et al., 2014). Currently, three meta-analyses have shown that female fitness is typically not maximized with a single mating (Arnqvist & Nilsson, 2000; Slatyer et al., 2012; South & Lewis, 2011). As such, female optimal mating rates, at least in some species, are likely higher than previously assumed which can have major implications for the extent to which the sexes are in conflict over mating rates and the strength of sexual selection operating on females.

Over the course of my doctorate studies, I carried out several experiments to extend our knowledge of sexual conflict from the female perspective, bridge our understanding of

sexual conflict with social behaviour, and quantify the fitness consequences of polyandry for females. My dissertation additionally introduces novel experimental approaches for using social network analysis as a tool to advance our understanding of animal social and sexual dynamics. In the next sections, I outline the major areas of my research and discuss the prior literature that motivated my questions.

1.2 Sexual Conflict

Sexual conflict is ubiquitous throughout the animal kingdom and plays a major role in the evolution of sex-specific morphological and behavioural traits that can provide benefits to one sex at the detriment of the opposing sex (Chapman et al., 2003; Parker, 1979, 2006). For example, since the optimal mating rate is typically higher in males compared to females (Janicke et al., 2016), males across taxa have evolved anatomical structures like elaborate claspers (Arnqvist, 1989b; Arnqvist & Rowe, 2002; Ng & Kopp, 2008) and grasping appendages (Crudgington & Siva-Jothy, 2000b; Friesen et al., 2013; Perry & Rowe, 2015) that enhance their ability to secure and prolong matings with resistant females. Behaviourally, males across species are known to engage in various coercive tactics like sexual harassment which involves securing mating opportunities through the constant pursuit, chasing, or mounting of females (Baniel et al., 2017; Dukas & Jongsma, 2012; McKinney & Evarts, 1997; Partridge & Fowler, 1990; Saveer et al., 2021). In response, females are expected to evolve adaptations that defend against such harmful male traits which can ultimately result in a co-evolutionary arms race between the sexes (Arnqvist & Rowe, 2002; Chapman, 2006; Perry & Rowe, 2012). Some female resistance traits have been characterized like the spermelege in female bed bugs (*Cimex lectularius*) that reduce the costs of traumatic insemination (Morrow & Arnqvist, 2003) and the abdominal spines of female water striders (*Gerris incognitus*) that hinder grasping by males (Arnqvist & Rowe, 1995). Yet, well-characterized examples of how females resist male sexual strategies remain lacking, even after several reviews have called for an increased focus on the female side of sexual conflict (Arnqvist & Rowe, 2005; Chapman, 2006; Fricke et al., 2009; Perry & Rowe, 2015).

Behaviourally, females can avoid costly mating and harassment through pre-mating struggles that involve wrestling or thrashing (Rowe, 1992; Watson et al., 1998), running away from males (Killen et al., 2016; McKinney & Evarts, 1997), or choosing to remain both physically and socially distant from males for extended periods of time (Dadda, 2015; Darden & Croft, 2008; Stanley et al., 2018). However, while reducing superfluous matings may be beneficial, behavioural avoidance of males can also come with energetic or opportunity costs that females must account for in order to maximize their fitness. A clear illustration of this trade-off can be found in water striders (*Gerris remigis*) where females can reduce harassment from males by sacrificing foraging opportunities (Krupa et al., 1990). Given the existence of these trade-offs, it is still unclear as to whether, when, and how females should behaviourally avoid males and if female responses vary under different contexts. Because life-history traits like mating history or social experience are known to influence the expression of various behaviours (Baxter & Dukas, 2017; Crews et al., 1997; Dukas, 2004, 2005; Harlow et al., 1965; Taborsky et al., 2012), they may also modulate how females respond to harassment thus influencing sexual interactions more broadly.

In addition to driving the evolution of sex-specific traits, sexual conflict can also dramatically influence the social interactions of animals. In general, living in groups comes with many advantages such as increased mating opportunities, access to social information, sharing of resources, and communal vigilance against predators (Krause et al., 2002; Ward & Webster, 2016). However, group-living also increases the likelihood of antagonistic interactions between the sexes. In fact, a handful of studies have found that high levels of harassment from males can cause females to engage in various forms of social avoidance, resulting in segregation of the sexes (Darden et al., 2009) or more disparate social networks (Darden & Croft, 2008). Yet, most studies on social behaviour still do not consider the potential influences that sexual conflict can have on social decision-making and the composition of social groups.

Using bed bugs (*Cimex lectularius*) as model organisms, I carried out several experiments examining how females behaviourally avoid costly interactions with males and the social ramifications of sexual conflict. Bed bugs are one of the most frequently

cited examples of sexual conflict as they obligately reproduce via traumatic insemination where males use their needle-like intromittent organ to pierce the female abdomen and insert sperm (Carayon, 1966; Reinhardt & Siva-Jothy, 2007; Stutt & Siva-Jothy, 2001). Traumatic insemination in bed bugs appears to involves a fitness cost to females owing to injury and the energetic costs of wound-healing (Siva-Jothy et al., 2019; Stutt & Siva-Jothy, 2001). Thus, excessive inseminations should be avoided by females. Anatomically, female bed bugs have evolved a region of thickened cuticle known as the spermelege which reduces the damaging effects of traumatic insemination (Morrow & Arnqvist, 2003). However, apart from the documentation of two refusal postures (Saveer et al., 2021; Siva-Jothy, 2006), one of which only occurs after prolonged starvation, little is known about how females behaviourally respond to threats of costly insemination. In addition to being known for their sexual conflict, bed bugs also exhibit social behaviour. They are typically found in mixed-sex aggregations within protective crevasses and emit chemical and tactile cues to facilitate social attraction (Johnson, 1941; Mellanby, 1939; Reinhardt & Siva-Jothy, 2007; Siljander et al., 2007, 2008). Combined, these sexual and social features make bed bugs an ideal model for studying the complex interplay between sexual conflict and social behaviour.

1.3 Animal Social Networks

Recent applications of network analysis to non-human animal studies have demonstrated its utility for testing hypotheses about the ecological and evolutionary pressures that shape animal social and sexual dynamics (Croft et al., 2008, 2011; Farine & Whitehead, 2015; Pinter-Wollman et al., 2014; Sih et al., 2009). Within a network framework, individual animals are represented as nodes while connections between nodes, known as edges, can then be used to represent affiliative or antagonistic associations and interactions. For example, edges are often used to represent social associations which can be based on observations of proximity between individuals or shared space use (Farine & Whitehead, 2015). Edges can also be used to represent distinct interactions amongst individuals like grooming (Crailsheim et al., 2020; Testard et al., 2021), fighting

(Tregenza et al., 2019), or mating (Greenway et al., 2021; McDonald et al., 2017, 2019b). Once a social network has been constructed, researchers can then extract a variety of metrics that describe the connections at either the level of the individual or at the level of the entire network.

Most social network studies in behavioural ecology have either been purely descriptive or observational. Such descriptive studies have generated several novel insights about the importance of indirect connections (Brent, 2015), individual variation in social connectivity (Pinter-Wollman et al., 2014), and the fitness correlates of such individual variation (Blumstein et al., 2018; Formica et al., 2012). An increasing number of research groups, however, have begun to develop and adopt various methods of combining social network analysis with an experimental framework to achieve greater explanatory power. For instance, Stroeymeyt et al., (2018) showed that in response to controlled pathogen exposures, ant colonies (*Lasius niger*) will exhibit marked segregation between potential disease sources and high-value individuals, thereby demonstrating that complex animal societies can plastically and adaptively modulate their social network structure. In another experimental network-based study, researchers generated small populations of fruit flies that differed in their social history and illustrated that groups comprised of socially raised as opposed to socially isolated individuals formed more distinct social clusters and stable subgroups (Bentzur et al., 2021). Given that social network analysis has been shown to be useful for assessing responses to sexual harassment (Darden et al., 2009), the operation of sexual selection (McDonald & Pizzari, 2017), and the consequences of extreme polyandry (Greenway et al., 2021), taking an approach that blends social network analysis with experimental manipulations can serve as a powerful approach for examining the interplay between sexual conflict and social behaviour in complex group environments.

1.4 The Female Fitness Consequences of Polyandry

Following the growing recognition that polyandry is widespread throughout the animal kingdom, considerable effort has been put towards elucidating the fitness benefits and costs

that females accrue from mating with multiple males (Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000; Kokko & Jennions, 2023; Parker & Birkhead, 2013; Simmons, 2005; Slatyer et al., 2012; Snook, 2014). The potential benefits of polyandry for females can be divided into two major sub-categories: direct benefits and indirect benefits. Direct benefits encompass material resources acquired through mating that increases females' lifetime reproductive success (Arnqvist & Nilsson, 2000; Ridley, 1988; Snook, 2014). Currently, the most empirically supported direct benefit of polyandry is the acquisition of nutritious substances like nuptial gifts or seminal fluid compounds that aid in females' egg and offspring production (Arnqvist & Nilsson, 2000; South & Lewis, 2011). However, other potential direct benefits of polyandry include fertility assurance, increased paternal care, and a reduction of harassment (convenience polyandry) (Arnqvist & Nilsson, 2000; Boulton et al., 2018; Ridley, 1988; Snook, 2014). Indirect, or genetic, benefits encompass instances where mating with multiple males increases the average fitness of a female's offspring. For example, acquiring sperm from multiple males could increase the genetic diversity or competitiveness of a female's offspring (Simmons, 2005; Slatyer et al., 2012; Yasui, 1998, 2001; Yasui & Garcia-Gonzalez, 2016). However, compared to direct benefits, fewer studies have empirically assessed the indirect benefits of polyandry due to the enormous effort required to measure the fitness of experimental females' offspring. As a result, asides from some evidence from birds that extra-pair copulations can help females secure better genes through "trading up" (Møller, 1990, 1992) and some evidence from arthropods showing that mating with multiple males can increase egg viability (Simmons, 2005; Slatyer et al., 2012), the extent to which females gain indirect benefits from polyandry remain unresolved.

While polyandry can provide females with several benefits, there are also well-documented costs of mating for females. For example, mating is often associated with time and energy costs (Watson et al., 1998), increased predation rates (Rowe et al., 1994), reduced foraging efficiency (Rowe, 1992; Stone et al., 1995), exposure to disease/parasites (Jennions & Petrie, 2000), decreased longevity due to harmful ejaculate substances (Chapman, 2001), and risk of injury or death (Baniel et al., 2017; Johns et al., 2009).

Optimal female mating rates therefore will depend on the balance between the benefits and costs of accepting or acquiring each additional mate. It has been suggested that females exhibit an optimal intermediate mating rate since the benefits of mating multiply are hypothesized to diminish as females mate with an increasing number of males while the costs are predicted to be additive (Arnqvist & Nilsson, 2000). However, despite a growing interest in the consequences of female multiple mating and sexual selection on females over the past few decades, few critical experiments have been conducted to test this optimal polyandry hypothesis.

1.5 Structure of the Thesis

In this section, I will provide a brief overview of the next four data chapters of my dissertation in relation to my overall research goals.

I began my graduate studies with the goal of examining how sexual conflict can influence social behaviour and specifically focused on whether females socially avoid males to mitigate harassment. Therefore, in Chapter 2, I constructed a novel semi-naturalistic arena to examine potential sex differences in the tendency to form social aggregations and whether aggregations were significantly assorted by sex. I found no evidence of decreased female sociality or females preferentially aggregating with other females when placed in a realistic group setting. As a follow-up, I tested the social preferences of females individually, which revealed a strong preference for female rather than male social cues. Taken together, my results suggest sexual conflict over the social environment. While performing these experiments, I documented high rates of polyandry with females mating roughly once per day. I also noted several instances of females running away from males. These observations directly informed my questions and experiments in Chapter 3.

For Chapter 3, I sought out to understand how the observed rates of traumatic insemination in semi-naturalistic settings affects female fitness. Additionally, I wanted to formally document and better understand the factors that influence female avoidance behaviour. After showing that daily traumatic insemination rates fall closer to the male

rather than female optima, I critically tested whether females' insemination status influences their propensity to run away from males in a realistic social network setting. In a third experiment, I wanted to better understand the relationship between female mating history and avoidance behaviour and therefore video-recorded female bed bugs as they received daily inseminations over six consecutive days.

In Chapter 4, I continued to investigate the interplay between social and sexual dynamics, this time focusing on how social experience shapes males' abilities to secure inseminations as well as females' abilities to avoid excessive inseminations. I achieved this my generating socially isolated vs. socially experienced bed bugs and directly pit individuals from both treatments against each other in a complex and competitive group environment.

Finally, in Chapter 5, I used fruit flies (*Drosophila melanogaster*) to test whether females exhibit an optimal intermediate rate of polyandry. A major goal of this chapter was to subject females to mating rates that reflect polyandry in nature as opposed to the lower rates of polyandry that have been tested in previous studies. Through controlled mating trials, I exposed females to either a low (every eight days), medium (every four days), or high (every two days) mating rate while limiting and controlling for females' exposure to sexual harassment. I found that even at more realistically high rates, polyandry can lead to net fitness benefits for females which can have major implications for the evolution of secondary sex characteristics in females and sperm competition amongst males.

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CHAPTER 2 – THE SOCIAL CONSEQUENCES OF SEXUAL CONFLICT IN BED BUGS: SOCIAL NETWORKS AND SEXUAL ATTRACTION

Yan, J.L., Dukas, R. (2022). The social consequences of sexual conflict in bed bugs: Social networks and sexual attraction. *Animal Behaviour,* 192, 109-117.

2.1 ABSTRACT

Sexual conflict is ubiquitous across the animal kingdom and often involves costly sexual harassment of females by males. An overlooked outcome of sexual conflict is its potential impact on social behaviour. Due to their seemingly harmful mode of copulation, traumatic insemination, and tendency to form aggregations, bed bugs are an ideal model for studying the social implications of sexual conflict. Repeated traumatic inseminations are known to reduce some aspects of female fitness so we thus expected the benefits to males but high costs of frequent mating for females to result in divergent social preferences between the sexes. To examine the impact of sexual harassment on social structure, we devised a novel experimental arena with either 2 or 12 shelters and continuously tracked sexual and social interactions between individually marked bed bugs over six days. By constructing aggregation networks, we examined whether female bed bugs occupied more peripheral network positions compared to males as well as whether females preferentially associated with other females as a strategy to reap the benefits of group-living while mitigating the costs of unsolicited sexual attention. We found no evidence that females were shaping their social environment to evade associating with males. However, when tested individually in a follow-up experiment, mated females showed a strong preference for social cues from females over social cues from males. Our results therefore suggest that males and females may be in conflict over the composition of social associations and highlight the importance of both examining behaviour at the individual level and tracking larger groups of freely interacting populations in more complex environments.

2.2 INTRODUCTION

The past two decades have seen a gradual shift in our perception of animal social behaviour with the growing appreciation that many species traditionally considered solitary possess complex social lives. Individuals of the apparently solitary species clearly do not live in integrated social groups such as social hymenopterans (Kapheim et al., 2015; Michener, 1974; Seeley, 2010; Wilson, 1971), social mammals (Cheney & Seyfarth, 2008; Clutton-Brock, 2016; Sherman et al., 1991) and cooperatively breeding birds (Brown, 1987; Koenig & Dickinson, 2004). Nevertheless, numerous "solitary" species have parental care that involves an extended period of life within a group, aggregation pheromones that bring together dispersed individuals, food sharing, and communal anti-predatory behaviours modulated via alarm pheromones (Caro, 1994; Costa, 2006; Elbroch et al., 2017; Prokopy & Roitberg, 2001; Wertheim et al., 2005).

While there are clear advantages to life in groups, the close proximity to other individuals also increases the opportunity for a variety of antagonistic interactions. One source of such tension is sexual conflict, which occurs when the reproductive interests of the two sexes are at odds with one another (Chapman, 2006; Parker, 1979). This conflict is pervasive among sexually reproducing animals and often results in sex-specific behaviours and adaptations that provide benefits to one sex at the detriment of the opposing sex (Chapman et al., 2003). A common manifestation of sexual conflict is sexual harassment, where males pursue females through coercive tactics to gain access to reproductive opportunities (Parker & Clutton-Brock, 1995). Well-documented costs of sexual harassment to females include physical injury (Baniel et al., 2017), reduced foraging efficiency (Pilastro et al., 2003; Stone set al., 1995), and increased predation rates (Arnqvist, 1989). All these costs can decrease female fitness (den Hollander & Gwynne, 2009; Dukas & Jongsma, 2012; Sakurai & Kasuya, 2008). Consequently, females of many species have evolved physiological, morphological, and behavioural strategies for evading harmful male pursuit (Brennan et al., 2007; Crudgington & Siva-Jothy, 2000; Lessells, 2006; Morrow & Arnqvist, 2003; Siva-Jothy et al., 2019).

Most studies on social behaviour do not consider sexual conflict, and much of the research on sexual conflict does not address its ramifications for the evolutionary biology of social behaviour. There are, however, tight interactions between the two disciplines because living in groups increases the opportunities for antagonistic interactions between males and females, and such sexual conflict can reduce the benefits that females incur from living in groups. Indeed a few studies indicate that male harassment causes females to engage in social avoidance. For example, in response to sexual harassment, female water striders (Aquarius remigis) reduce their activity in the center of experimental pools where large numbers of males are found and instead, spend most of their time on the edge of pools and out of water (Krupa & Sih, 1993). Likewise, in the Trinidadian guppy (Poecilia reticulata), exposure to male harassment drives females to select lower-quality habitats leading to segregation of the sexes (Darden & Croft, 2008), and results in females forming more disparate social networks (Darden et al., 2009). Other behavioural avoidance strategies include altering social distance from conspecifics (Dadda, 2015) and forming strategic alliances with more females or dominant males to shield oneself from unwanted male attention (Fox, 2002; Martens & Rehfeldt, 1989). Overall, these behavioural responses to harassment have the potential to critically influence social dynamics and the structure and composition of social groups.

The studies just noted suggest that the interdependent dynamics of social behavior and sexual conflict deserves further investigation. To this end, we used bed bugs (Cimex lectularius), a species often cited as an extreme model of sexual conflict as they have obligate traumatic insemination. During traumatic insemination, males use their needle-like copulatory organ to pierce through females' abdomens and deposit sperm directly into the body cavity (Carayon, 1966). Although traumatic insemination is relatively rare, it has evolved independently several times within invertebrates. Its benefits to males may be related to sperm competition (Lange et al., 2013; Tatarnic et al., 2014). In bed bugs, repeated traumatic inseminations have been shown to reduce female longevity and lifetime reproductive output likely due to the energetic costs of wound healing and increased frequency of infection (Stutt & Siva-Jothy, 2001).

Bed bugs show moderate social behaviour. In natural infestations, they are typically found in mixed sex aggregations within protective crevasses (Johnson, 1941; Reinhardt & Siva-Jothy, 2007). Their social attraction is driven by volatile and non-volatile chemicals as well as tactile cues (Gries et al., 2015; Reinhardt & Siva-Jothy, 2007; Siljander et al., 2007, 2008). Finally, bed bugs emit an alarm pheromone in response to cues of danger, and this leads nearby bed bugs to disperse (Levinson et al., 1974). The social and sexual features of bed bugs provide us with a unique opportunity to study how the presence of intense sexual conflict and harassment differentially affect the social tendencies of the two sexes, and how these differences are reflected at the population level.

To track the social and sexual dynamics of bed bugs, we developed a novel naturalistic arena, which allowed us to continuously observe populations of freely interacting bed bugs over several days. We experimentally manipulated the intensity of sexual conflict by providing the bed bugs with either 2 or 12 shelters. We expected females to experience higher rates of sexual harassment and traumatic insemination when given 2 shelters rather than 12 shelters due to the limited opportunities for avoiding males. Furthermore, we predicted that in the 12-shelter treatment, females would take advantage of the large number of shelters to employ male avoidance strategies. To detect patterns of female social avoidance, we used social network analysis, a powerful toolkit of statistical and graphical techniques used to analyze and visualize social relationships (Croft et al., 2008; Webber & Vander, 2019; Whitehead, 2008), to create networks based on how often we observed individuals in the same aggregation. First, we predicted that females would evade unwanted sexual advances from males by occupying less central network positions in these aggregation networks and by exhibiting lower levels of sociality overall, as quantified by their network strength. Second, we predicted that the bed bugs would show phenotypic assortment by sex, because preferentially associating with females would allow females to gain the benefits of aggregation without enduring the increased costs of harassment and traumatic insemination by males.

Our results indicated lesser tendencies than we expected of bed bugs to form aggregations when provided with many shelters, and no evidence for social avoidance by

females. Hence we conducted a follow up experiment to critically test bed bugs' specific social attraction to and avoidance of conspecifics of distinct sex and mating status. We allowed each focal bed bug to choose between two shelters that varied in their occupation history. First, as a baseline, we verified that both males and females would strongly prefer shelters previously occupied by females over shelters that had never harboured bed bugs. Second, we expected that previously mated males and females would prefer shelters formerly occupied by females over shelters previously occupied by males. This is because males should be highly attuned to cues that indicate potential mating opportunities, while females should avoid males owing to costly harassment and traumatic insemination. Finally, we predicted that males would prefer shelters previously occupied by virgin females over shelters formerly harbouring mated females. This could be owing to either mated females suppressing the emission of aggregation cues as a social avoidance strategy, or males' acute sensitivity and preference for virgin over mated females.

2.3 METHODS

Ethics Statement

Our research complied with all applicable laws and did not require approval from an ethics committee.

2.3.1 Study population and maintenance

We used descendants of bed bugs *(Cimex lectularius)* collected from four sites in Southern Ontario between October 2019 and January 2020. We maintained the colony in a small room kept at 27 ± 0.5°C at 40% relative humidity with lights off at 9:00 AM and on at 5:00 PM. We housed bed bugs in 240mL spice jars containing strips of folded filter paper to provide a rough surface for walking and oviposition. Each jar contained roughly 50 to 150 bed bugs of the same life stage. We fed the colony weekly under red light with defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using a Hemotek membrane-feeding system (Discovery Workshops, Accrington, UK).

2.3.2 Arena design and treatments

To observe sexual and social dynamics, we constructed a 34.5 cm x 23.5 cm x 15cm plexiglass arena with a 3 cm diameter circular hole cut into one of the shorter ends to perfectly fit a Hemotek feeding reservoir (Fig. 2.1a). To prevent escape, we secured a layer of mesh fabric that bed bugs could feed through over the feeding hole. We lined the arena floor with filter paper and further prevented escape by applying a layer of Fluon to the walls.

We manipulated shelter availability with two treatments, a 2-shelter treatment, which limited opportunity for social avoidance, and a 12-shelter treatment, which provided ample opportunity for female behavioural avoidance strategies (Fig. 2.1b). The choice of 2 and 12 shelters was based on our preliminary observations that 2 shelters could readily accommodate 24 bed bugs while 12 shelters provided sufficient opportunities for social avoidance. On average, shelters in the two treatments were at a similar distance from the blood source. Shelters were constructed from 5 cm x 7 cm x 0.3 cm balsa wood slat segments covered with glass microscope slides. Each segment of balsa wood contained two shelters created by cutting 1.5 cm x 3 cm cavities, each with a narrow 0.5 cm entrance. Each of these shelters were sufficiently spacious to accommodate all 24 adult bed bugs included in each replicate. In the 2-shelter treatment, shelters were placed in the center of the arena while for the 12-shelter treatment, the six segments of balsa wood were evenly spread out in the arena (Fig. 2.1b). We ran three replicates of each treatment.

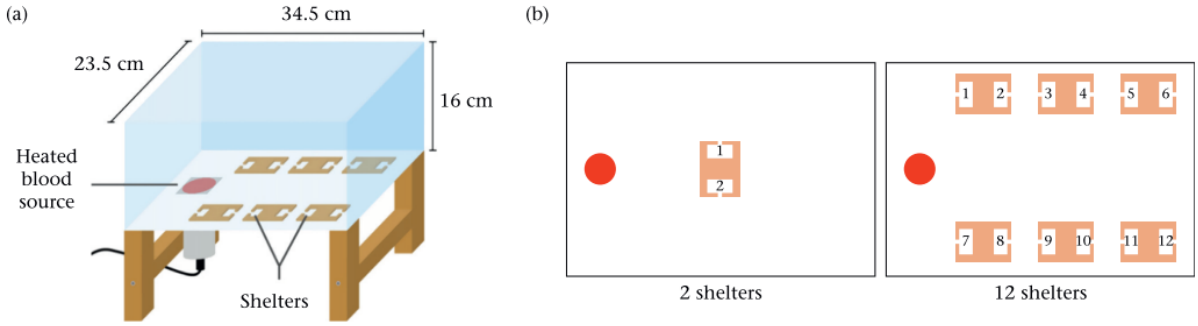

Figure 2.1. (a) Schematic overview of the Plexiglas experimental arena with dimensions. (b) Diagrams depicting overhead views of how shelters were arranged for the two-shelter and 12-shelter treatments.

2.3.3 Behavioural observations

For each replicate, we continuously observed 24 virgin, adult bed bugs (12 males, 12 females) for 24 hours a day over six consecutive days. We collected virgin focal individuals by isolating recently fed fifth instar juveniles until they emerged as adults. We then placed the newly emerged adults in same-sex groups and individually marked each bed bug with Sharpie oil-based paint markers. We released the focal individuals at the center of the arena 30 minutes before the start of the dark phase (8:30 am). The focal bed bugs typically remained highly active during the first couple of hours and explored much of the arena before settling into their shelters. We provided heated blood between 1:00 pm – 3:00 pm on the first, third, and fifth days of each replicate and stimulated foraging behaviour by exhaling into the arena at the beginning of each feeding period. All focal individuals fed at least once during the experiment. Throughout the dark period (9:00 am – 5:00 pm), when much of the bed bug activity occurs, we live-observed the bed bugs under red light and recorded all instances of mounting and traumatic insemination. Then, using a Canon VIXIA HF R800 camera, we video-recorded the bed bugs during the light period, and later scored from the videos all mountings and inseminations that occurred during the light period. Overall, we were able to determine the identities of both bed bugs for 2271 out of 2286 mountings and all 319 inseminations that occurred during the dark period and 344 out of 355 mountings and 45 out of 46 inseminations that occurred during the light period.

Mounting and traumatic insemination are highly stereotyped and distinctive behaviours. A mount consists of a male "jumping" onto a female and then dismounting within 5 seconds (Stutt & Siva-Jothy, 2001). An insemination is characterized by the male mounting the female and then remaining securely attached with his abdomen curled underneath the female's right abdomen (Carayon, 1966). Carayon (1966, p. 103) noted that inseminations last 1-5 min and Siva-Jothy and Stutt (2003, Fig. 2) depicted insemination durations of 30-300 s. In a data set including 193 insemination durations recorded in our laboratory for another experiment, the average ±1 SD insemination duration was 102.4 ± 53.9 s and the range was 18-406 s. Based on the literature, and because only two inseminations in our data set lasted less than 30 s, we chose 30 s as the minimum duration for a mounting to be considered insemination.

To validate our insemination criterion, we compared offspring production in two groups of 25 virgin, recently fed, 7-day old females. Each female of the inseminated group received a single traumatic insemination, while each female of the no insemination group did not interact with males. We then held all the females individually inside 35 mm petri dishes lined with filter paper. While 92% of the once-inseminated females produced eggs and hatchlings, no female of the no insemination group laid eggs.

As for social associations, we carried out scans at the start of each hour during the dark phase for a total of nine scans per day, where we documented the location and social partners of each bed bug. We considered bed bugs to be aggregated based on whether two individuals were touching or in a group of continuously touching bed bugs. We excluded one female and one male from two different 12-shelter replicates from our analyses as both bed bugs died within the first day of the experiment. One additional male from a 2-shelter replicate was removed from the analyses due to both behavioural and physical abnormalities – the male was unable to properly mount females and we later observed under a microscope that it had deformed genitalia.

2.3.4 Social network analyses

We created all network visualizations and ran our analyses with R version 4.1.1 (R Core Team, 2021). Using the *igraph* package (Csardi & Nepusz, 2006), we constructed social networks where weighted edges represented association indices between dyads based on how often they were observed in the same aggregation. Specifically, we used the simple ratio index (SRI) to calculate association indices, which is recommended for when nearly every individual can be reliably recorded in every sampling period (Hoppitt & Farine, 2018). Then, to quantify individual sociability, we extracted strength values from the aggregation networks. Strength is equivalent to the sum of all edge weights connected to a node and represents how often and with how many others an individual bed bug was seen aggregating with.

2.3.5 Statistics

We analyzed linear mixed-effects models (LMMs) in R using the package *lme4* version 1.1-27.1 (Bates et al., 2015) and report Wald χ^2 values generated with the *Anova* function from the *car* package version 3.0-11 (Fox & Weisberg, 2019). We verified model fits by visually inspecting plots of model residuals using the *DHARMa* package (Hartig, 2019). To examine whether the 2-shelter treatment resulted in higher levels of sexual harassment compared to the 12-shelter treatment, we constructed two LMMs, one with mounting rate and the other with insemination rate as the dependent factor. Both models included treatment as a fixed factor and replicate as a random factor.

We tested whether males were more social than females within each treatment using an LMM combined with a permutation test. In this model, we used the log of strength values taken from aggregation networks as the dependent factor and included treatment, sex, and the treatment by sex interaction term as fixed factors and replicate as a random factor. Because measures obtained from social networks are inherently non-independent, thus violating the assumptions underlying most parametric tests (Croft et al., 2011), we performed node-label permutation tests by shuffling and redistributing the nodes among all possible node positions in each of our six observed networks. This is a commonly used

approach for assessing whether nodes with different attributes reliably occupy different network positions (central/more social vs. peripheral/less social) (Farine & Whitehead, 2015). After obtaining new strength values from the randomized networks, we re-ran our LMM and extracted t-ratios from the relevant contrast using the package *emmeans* function in R. By performing 1000 iterations of this network randomization process, we were able to compare observed contrast t-ratios to a null distribution of t-ratios representing the null hypothesis that males and females do not differ in their propensity to aggregate. In total, we ran two permutation tests, one for male vs. female strength in the 2-shelter treatment and one for male vs. female strength in the 12-shelter treatment.

To examine whether the six bed bug populations showed positive assortment by sex, we calculated assortativity index (AI), a value between -1 and 1 where 1 represents perfect assortativity, -1 represents disassortativity, and 0 indicates no assortment, for each of the six aggregation-based networks. This was done using the *assortnet* package, which accounts for weighted edges (Farine, 2014). We then performed 1000 iterations of a node-label permutation test for each of the six observed networks. This resulted in a distribution of 1000 new AI's for each of our six bed bug populations representing the null hypothesis that associations between individuals were random or not biased by sex. We obtained two-tailed p-values by comparing the observed AI's for each network to its respective null distribution of AI's.

2.3.6 Social attraction experiment

To directly assess bed bugs' specific social attraction to conspecifics of distinct sex and mating status, we conducted a follow-up experiment with five treatments, where focal bed bugs could choose between two shelters that varied in their occupation history. First, as baseline control treatments, we presented either male or female focal individuals with the choice of a shelter previously occupied by mated females vs. an unused control shelter. Next, to test whether the social cues of males and females are differentially attractive to the two sexes, we presented either male or female focal individuals with the choice of a shelter previously occupied by mated females vs. a shelter previously

occupied by mated males. Lastly, to examine whether mating status alters attractiveness of females, we presented focal males with the choice of a shelter previously occupied by mated females vs. a shelter previously occupied by virgin females. We randomized and counter-balanced the position of the shelters and ran five replicates, each including six trials per each of the five treatments. Due to occasional shortages of bed bugs for generating scent cues, our final sample size was 29 trials per treatment except for the treatment of focal females choosing between cues of mated females and mated males, where we only had 28 trials.

We created choice arenas by placing two shelters at opposite ends of an 85 mm diameter petri dish, which was lined with filter paper and coated with Fluon around the side (Fig. 5). We constructed shelters by folding 15 mm x 15 mm segments of filter paper into triangular tents with floors, each held together by a small piece of masking tape. To manipulate their occupation history, we placed the shelters individually inside plastic vials 2.5 cm wide and 9.5 cm high, with four recently fed (< 2 hour) adult bed bugs. The bed bugs were either mated males, mated females, or virgin females. We obtained the mated females and mated males by collecting adult bed bugs of roughly the same age from our general population and virgin females by isolating recently fed fifth instar juveniles until they emerged as adults. We allowed these stimulus bed bugs four days to walk, rest, defecate, and lay eggs in and on the shelters. In a few cases, we used three (n = 7) or two (n = 3) stimulus bed bugs to generate social cues for each of the two shelter options instead of the usual four due to a shortage of age-matched bed bugs from the general population. Immediately before the choice assay, we immobilized the stimulus bed bugs using ice to remove them from the shelters. We ensured focal bed bugs were never housed in the same containers as bed bugs used to produce social cues to control for possible effects of familiarity.

For focal individuals, we generated virgin adult bed bugs as described above, then continued to keep the adults individually isolated for one additional week post-emergence. After this week of social isolation, we briefly consolidated the focal individuals in same-sex jars for feeding. The next day, we placed one male and one

female in a 50 mm petri dish lined with filter paper for up to ten minutes and verified that traumatic insemination had occurred using the same criterion as detailed above. Immediately after insemination, the pair of bed bugs were again isolated to ensure every bed bug had only mated once prior to the choice trial. At 1:00pm on the same day (the middle of the dark period), we placed the focals at the center of each petri dish. Twenty hours later, at the end of the light phase, an observer blind to treatment recorded the bed bugs' shelter choice.

We used the *lme4* package to perform generalized linear mixed-effects models (GLMMs). For the two control treatments, we ran a single binomial logistic regression with sex as a fixed effect and replicate as a random effect to assess whether attraction towards the used shelters varied by sex. We then ran a GLMM for each of the three other treatments again using the binomial distribution with replicate as a random factor to assess whether the bed bugs showed significant attraction to one type of social cue over the other.

2.4 RESULTS

2.4.1 Effect of shelter availability on harassment received by females

On average, females were mounted approximately 4.14 times a day and inseminated approximately 0.89 times a day. Females in the 2-shelter treatment were mounted more frequently compared to females in the 12-shelter treatment (LMM: Wald $X^2_1 = 26.58$, $p < 0.0001$; Fig. 2.2a). However, we did not detect any differences in traumatic insemination rates between the two treatments (LMM: Wald $X^2_1 = 0.73$, $p = 0.39$; Fig. 2.2b).

2.4.2 Male vs. female strength of aggregation

Overall, bed bugs of both sexes spent more time aggregating with conspecifics in the 2-shelter treatment compared to the 12-shelter treatment (LMM: Wald $X^2_1 = 336.74$, $p < 0.0001$; Fig. 2.3b). Within the 2-shelter treatment, females displayed higher levels of sociality compared to males ($p_{rand} < 0.01$; Fig. 2.3b; Fig. A2.1) while sex differences in

network strength were not detected in the 12-shelter treatment (p_{rand} = 0.13; Fig. 2.3b; Fig. A1b).

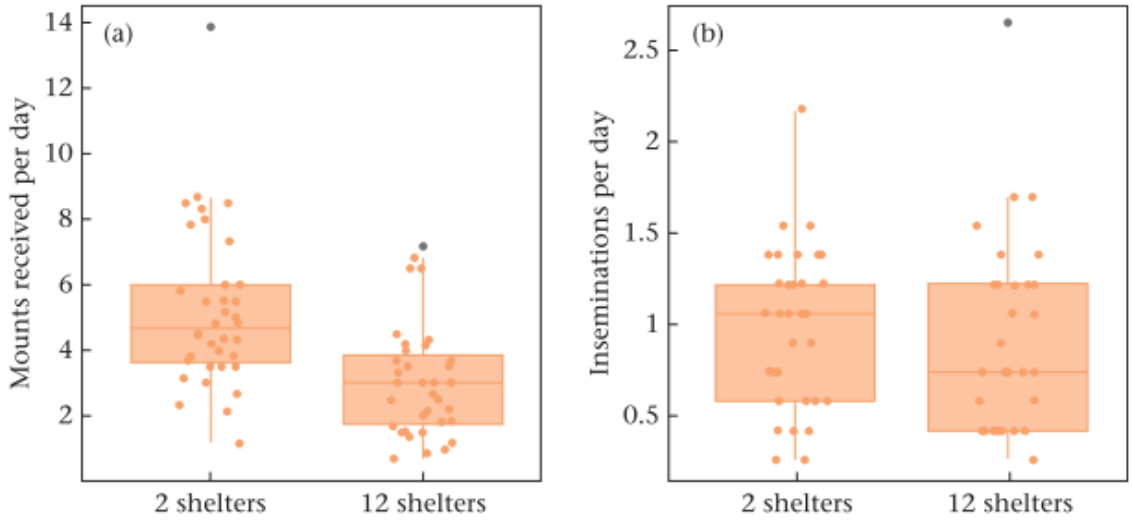

Figure 2.2. Effect of treatment on the amount of sexual harassment received by females. The daily rate of (a) mounts and (b) traumatic inseminations received by females in the two- shelter (N = 36) versus 12-shelter (N = 35) treatments. Bold horizontal lines indicate the medians, the boxes represent the interquartile range (IQR) between the first and third quartiles, and the vertical lines extend to the minimum and maximum values. Outliers are shown in black.

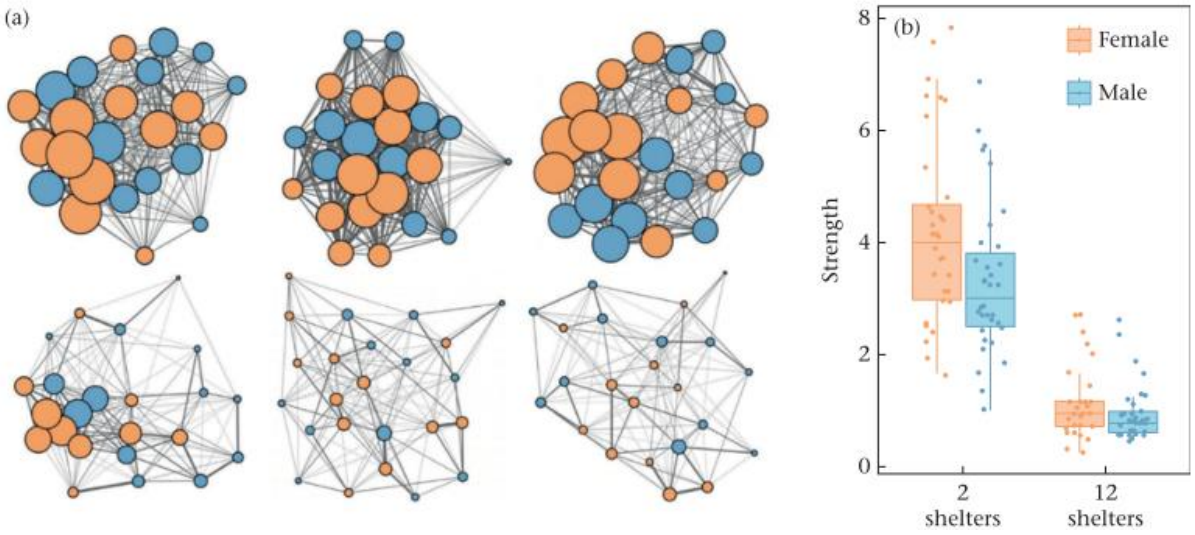

Figure 2.3. (a) Aggregation networks of bed bug groups. The top row represents networks from two-shelter treatment groups while the bottom row represents networks from 12- shelter network groups. Orange nodes denote females while blue nodes denote males. Edge width represents the strength of association between dyads and node size corresponds to strength (total sum of edge weights). For clearer visualization, node size for the second two-shelter network is scaled to half the size of nodes relative to all the other networks. (b) Strength comparison between males and females within each of the two treatments.

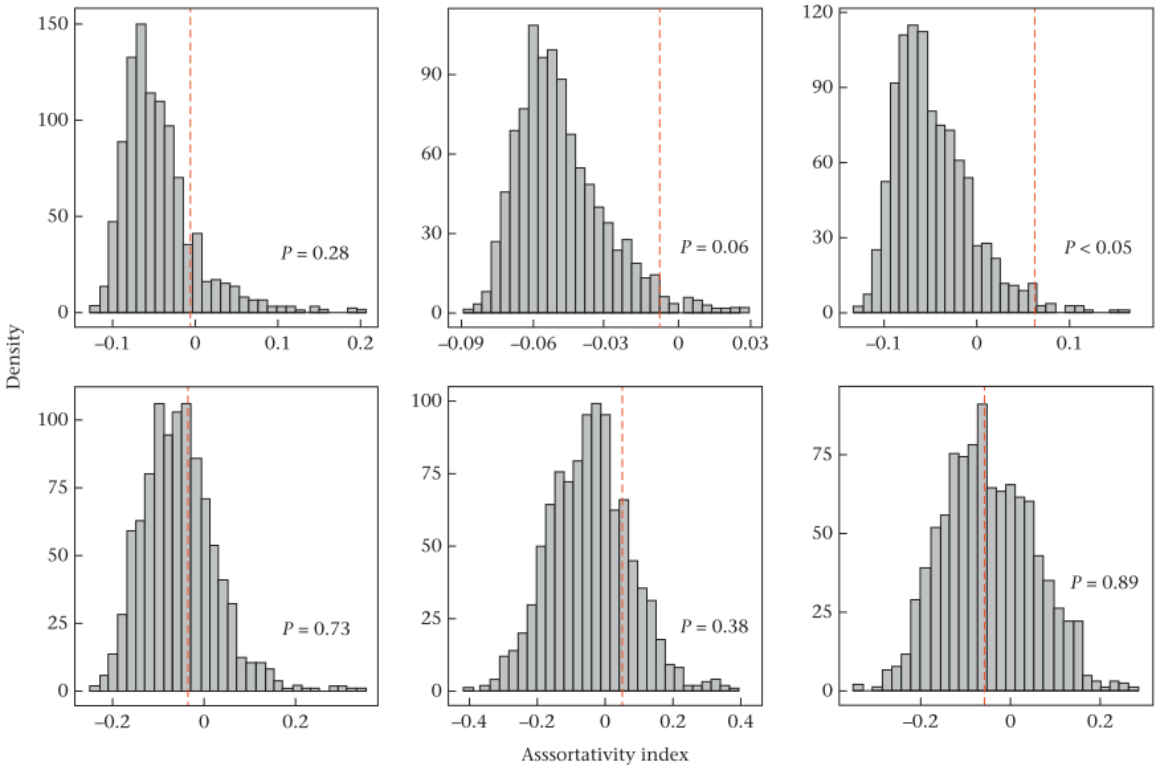

Figure 2.4. Distribution of assortativity indexes from permuted networks. The top row represents randomized networks from the two-shelter treatment while the bottom row represents randomized networks from the 12-shelter treatment. Red dashed lines represent assortativity indexes of the observed networks.

2.4.3 Assortativity by sex

In each of our six bed bug groups, observed assortativity indexes were close to zero, indicating no preference for aggregating with same sex vs. opposite sex individuals (Fig. 2.4). Additionally, our network randomization tests revealed that only one out of the six bed bug populations showed significant, though low, positive assortment ($p_{rand} < 0.05$; Fig. 2.4). The remaining five networks were not more assorted than one would expect by chance, indicating no significant tendency for individuals to aggregate with same or opposite sex conspecifics (Fig. 2.4).

2.4.4 Individual choice assays

Focal mated males and focal mated females both preferred shelters previously occupied by mated females over unused control shelters (GLMM intercept: Wald $X^2_1 = 11.20$, $p < 0.001$; sex: Wald $X^2_1 = 0.97$, $p = 0.32$; Fig. 2.5). When presented with the choice between shelters previously occupied by mated males and shelters previously occupied by mated females, focal males showed a non-significant tendency towards mated females (GLMM intercept: Wald $X^2_1 = 1.98$, $p = 0.16$; Fig. 2.5) while focal females significantly preferred mated females (GLMM intercept: Wald $X^2_1 = 9.56$, $p < 0.01$; Fig. 2.5). Lastly, when presented with the choice between shelters previously occupied by virgin females and shelters previously occupied by mated females, focal males preferred shelters with cues from virgin females (GLMM intercept: Wald $X^2_1 = 5.40$, $p < 0.05$; Fig. 2.5).

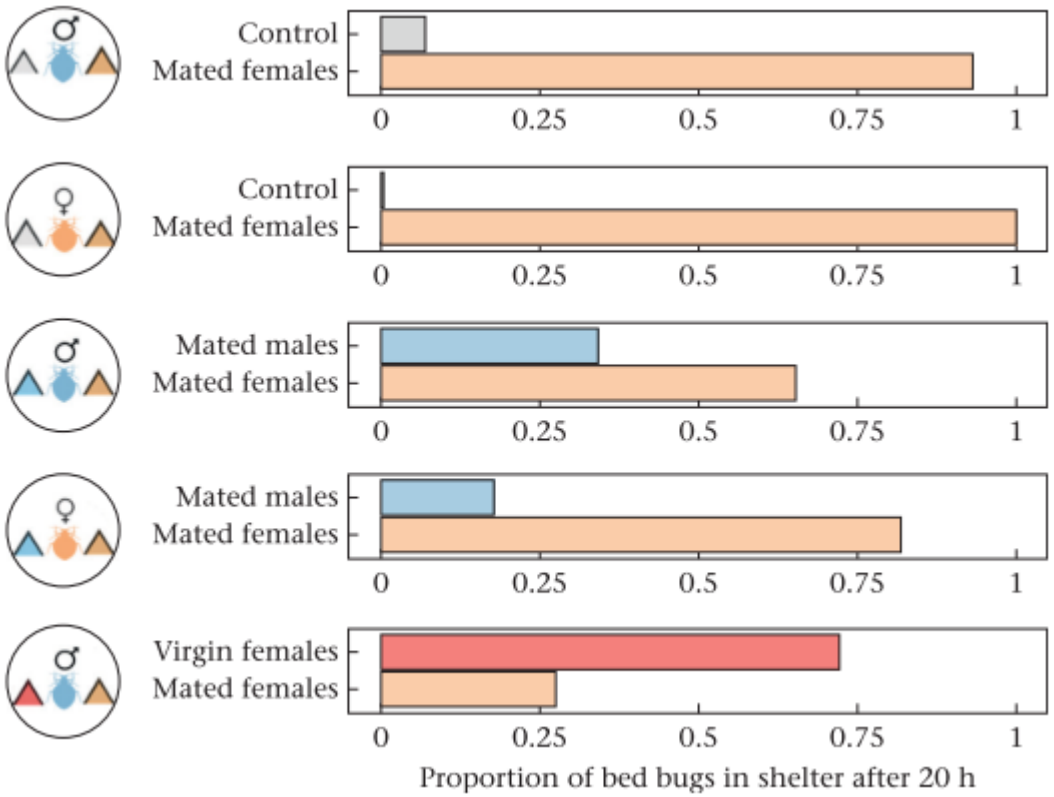

Figure 2.5. We gave individual focal adult bed bugs a binary choice between two filter paper shelters that varied in their occupancy history. Occupation history of shelters varied between the five treatments denoted by the colour of triangular shelters in each diagram, where blue represents mated males, orange represents mated females and red represents virgin females. The first two treatments included an unused control shelter as one of the two shelter options and is shown as a grey triangle. Bars correspond to the occupancy history of shelters; bed bug colours correspond to the sex of focal individuals, with blue representing mated adult males, orange representing mated adult females and red representing virgin females.

2.5 DISCUSSION

Using a novel semi-naturalistic arena, we tracked aggregation and traumatic insemination patterns of replicate bed bug populations over six consecutive days. As traumatic insemination in bed bugs is often cited as an extreme example of sexual conflict (Reinhardt & Siva-Jothy, 2007; Siva-Jothy, 2006; Stutt & Siva-Jothy, 2001), we constructed aggregation networks to assess whether we would see signs of social avoidance strategies used by females at the population level to avoid sexual harassment as seen in other species (Dadda, 2015; Darden & Croft, 2008; Krupa et al., 1990; Stanley et al., 2018). Contrary to our predictions, we found that females were not less social than males overall, and that social networks were not assorted by sex. The lack of observed female social avoidance patterns may suggest female bed bugs are well counter-adapted for mitigating potential costs of repeated inseminations as suggested by Morrow & Arnqvist (2003). Given the mixed empirical evidence on how harmful traumatic insemination is to females (Morrow & Arnqvist, 2003; Stutt & Siva-Jothy, 2001), additional research into the actual fitness consequences of different traumatic insemination rates is needed. Nonetheless, our fine-scale continuous observation of bed bugs revealed several novel insights about both their sexual and social dynamics.

First, we predicted that reducing shelter availability would dramatically increase sexual conflict intensity through sexual harassment, which we quantified using mounting and insemination rates. However, we found that only mounting (Fig. 2.2a), but not insemination rate (Fig. 2.2b), was higher in the 2 vs. 12-shelter treatment. Furthermore, our data revealed that the majority of mounts did not result in successful insemination (Fig. 2.2a, b). The high proportion of unsuccessful mounts suggest that insemination rate is not as male-controlled as previously thought (Reinhardt et al., 2009b; Stutt & Siva-Jothy, 2001). Accordingly, we often observed females running away from sexually harassing males or assuming a refusal posture as described by Siva-Jothy (2006). Thus, although our aggregation networks did not reveal patterns of female avoidance at the population level, our documentation of general avoidance behaviour highlights the importance of fine scale continuous observations as well as the importance of studying sexual conflict in more

complex, realistic environments, which allow females to perform their full range of evolved avoidance strategies.

Another key consideration and likely explanation for the lack of difference in insemination rate between our two treatments is sperm and/or seminal fluid constraint in males, which has been previously documented in a range of taxa including bed bugs (Birkhead, 1991; Linklater et al., 2007; Preston et al., 2001; Radhakrishnan et al., 2009; Reinhardt et al., 2011). Because male bed bugs are known to experience seminal fluid depletion and can gauge the recent mating history of a female using their copulatory organ (Siva-Jothy & Stutt, 2003), mounts that do not result in insemination could be the result of males adaptively aborting insemination attempts based on indicators of female attractiveness or potential sperm competition. Closer investigation of how males differentially pursue females that vary in traits such as recent mating history can reveal new insights on male mate choice, sexual selection, and mating system evolution.

Females did not utilise the increased number of shelters in the 12-shelter treatment to occupy more peripheral network positions to avoid males. Moreover, to our surprise, bed bugs in the 12-shelter treatment formed relatively sparse social networks with low strength values. That is, when given a choice among a dozen high quality shelters, the bed bugs did not form the anticipated large aggregations. Rather, the average group size was about two (Fig. 2.3a, b). This was unexpected because natural infestations of bed bugs typically comprise large, mixed-sex aggregations (Johnson, 1941; Mellanby, 1939; Reinhardt & Siva-Jothy, 2007). Furthermore, our social preference test revealed that both male and female adult bed bugs show a strong preference for occupying shelters with social cues from conspecifics over identical shelters with no social cues (Fig. 2.5), echoing results from previous studies on bed bug social attraction (Gershman et al., 2019; Levinson & Bar Ilan, 1971; Weeks et al., 2011, 2013). This apparent contradiction between our social network study and follow-up experiment could be explained by the absence in the arena of pre-existing physical and chemical stimuli including feces, exuviae, eggs, and pheromones, which may be crucial for facilitating aggregation formation in bed bugs.

As for the 2-shelter treatment, we found that females were more social than males (Fig. 2.3b; Fig. A2.1a). However, our networks alone cannot tell us if higher female strength values are the result of females themselves showing a higher propensity to seek others or if other individuals preferentially associate with females over males. With our social attraction experiment, we directly addressed this question and found that mated females strongly prefer shelters with cues from other females over other males and that males too, tended towards a preference for females (Fig. 2.5). Therefore, females occupying more central network positions in the 2-shelter treatment likely reflect a strong tendency for both females and males to associate with females over males. However, despite females' preference for shelters previously occupied by females as opposed to males, we still found that bed bug networks from both treatments generally showed no assortment by sex (Fig. 2.4). This suggests that females are incapable of engineering their social environment to reduce levels of sexual harassment, most likely because males are adept at locating and exploiting females even in a relatively large, complex environments.

Lastly, we found that males can discriminate between social cues left by virgin vs. mated females, with a preference for virgin females presumably because of their higher reproductive value (Fig. 2.5). This suggests that females adjust their deposition of contact pheromone based on their reproductive status as indeed suggested by Siljander et al. (2007). It also tells us that in addition to using their intromittent organ to directly assess a female's mating history (Siva-Jothy & Stutt, 2003), males also possess indirect mechanisms of assessing their reproductive landscape to strategically seek mating opportunities that lessen sperm competition intensity and thus increase reproductive success.

Overall, our semi-naturalistic social network experiment revealed that female bed bugs struggled to socially evade males even when provided with several high-quality shelters. Yet, at the individual level, females showed a clear tendency to avoid shelters with social cues from males. We thus conclude that female bed bugs are generally incapable of shaping their social environment in a way that reduces levels of sexual harassment. Further research taking a network-based approach on sexual and social dynamics can better

elucidate how competing reproductive interests can shape social behaviour at both the individual and population level.

2.6 ACKNOWLEDGEMENTS

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2.8 APPENDIX

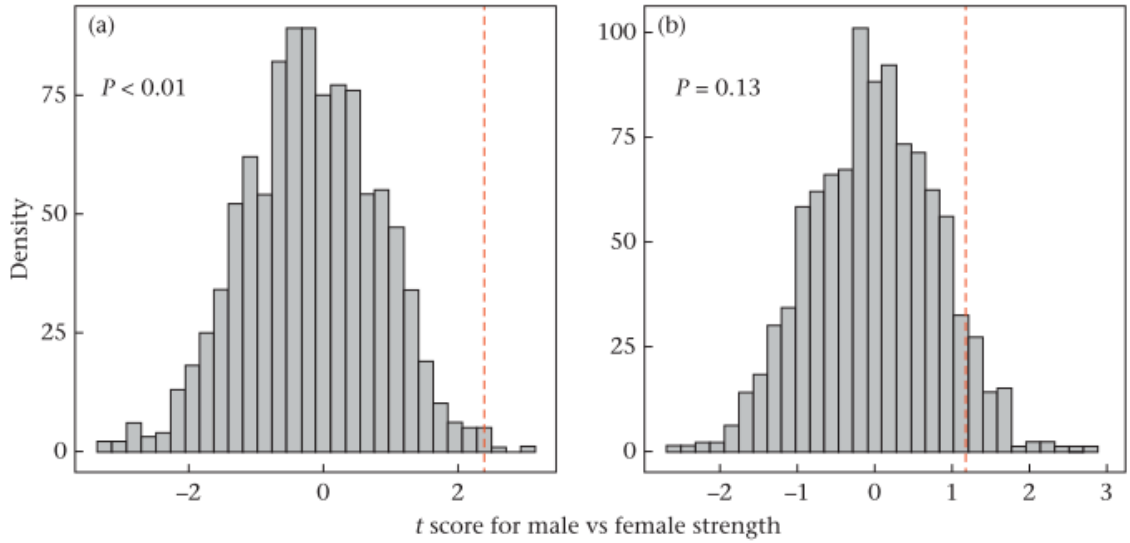

Figure A2.1. Null distributions of t scores representing the effect of sex on strength for (a) the two-shelter treatment and (b) the 12-shelter treatment. Null distributions for each treatment are the result of 1000 node-label network randomizations. Red dashed lines are t scores representing the observed effect of sex on strength for each treatment.

CHAPTER 3 – SEXUAL CONFLICT AND SEXUAL NETWORKS IN BED BUGS: THE FITNESS COSTS OF TRAUMATIC INSEMINATION, FEMALE AVOIDANCE AND MALE MATE CHOICE

Yan, J.L., Dobbin, M.L., Dukas, R. (2024). Sexual conflict and sexual networks in bed bugs: the fitness cost of traumatic insemination, female avoidance, and male mate choice. *Proceedings of the Royal Society B*. 291: 20232808.

3.1 Abstract

Sexual conflict is prevalent among animals and is primarily caused by the fact that the optimal mating rates are often higher in males than in females. While there is a growing appreciation that females can also gain from multiple matings, we still know relatively little about which sex controls the observed mating rates and how close it is to the optimal female mating rates. To address this issue, we tracked female bed bugs (*Cimex lectularius*) inseminated daily versus weekly and found that weekly inseminated females lived longer and produced over 50% more offspring. In a follow-up experiment employing a social network framework, we placed 24 bed bugs into a semi-naturalistic arena and recorded all sexual interactions. While recently inseminated females did not avoid males more often, they were more frequently rejected by males. Finally, we tracked avoidance behaviour in a single cohort of female bed bugs as they received six successive daily inseminations. Avoidance rates increased and insemination durations decreased with increasing number of prior inseminations. Overall, our results indicate high costs of polyandry. Although females possess some plastic avoidance strategies, the observed rates of insemination fall closer to the male rather than female optimum.

3.2 Introduction

Sexual conflict occurs when the reproductive interests of males and females are at odds with one another (Chapman, 2006; Parker, 1979). This conflict is prevalent among animals and manifested in sex-specific traits that provide benefits to one sex at the detriment of the

other (Chapman et al., 2003; Chapman, 2006; Parker, 2006). A common cause for sexual conflict is the fact that the optimal mating rate is typically higher in males than in females, which leads to males often pursuing and even coercing reluctant females into mating (Arnqvist & Nilsson, 2000; Bateman, 1948; Clutton-Brock & Parker, 1995). Examples of sexually antagonistic traits that benefit males at the expense of females include the elaborate morphological structures of male water striders (*Gerris odontogaster*) that have evolved for grasping resistant females (Arnqvist, 1989b; Arnqvist & Rowe, 2002) and seminal fluid proteins, which enhance male paternity share but decrease female survival (Chapman et al., 1995; Chapman, 2001; Civetta & Clark, 2000; Rice, 1996) . While sexual conflict is well documented in many species, there is now a growing appreciation that the optimal mating rates of females are higher than previously thought. Even though females may be able to produce offspring for the rest of their lives after a single mating, some intermediate mating rates may balance the costs and benefits to females and hence maximize their lifetime reproductive success (Arnqvist & Nilsson, 2000; Boulton et al., 2018; Jennions & Petrie, 2000; South & Lewis, 2011).

Although females may gain from multiple matings, we still know relatively little about their optimal mating rates and how they compare to naturally observed mating rates. If observed mating rates are determined by males, they may exceed the rates that are optimal for females. To address this issue, we conducted a series of experiments using bed bugs (*Cimex lectularius*) as a model system. Bed bug reproduction involves obligatory traumatic insemination, whereby males bypass females' genital tracts and instead use their needle-like intromittent organs to pierce female abdomens and insert sperm (Carayon, 1966; Reinhardt & Siva-Jothy, 2007; Stutt & Siva-Jothy, 2001; Tatarnic et al., 2014). Although traumatic insemination has independently evolved multiple times and is prevalent among a wide variety of animals, its evolutionary biology is not well understood (Lange et al., 2013; Tatarnic et al., 2014). Traumatic insemination in bed bugs involves a fitness cost to females owing to the injury and subsequent immune response and healing (Reinhardt et al., 2003; Siva-Jothy et al., 2019; Stutt & Siva-Jothy, 2001). Despite such costs and the fact that female bed bugs remain fertile for about 9.5 weeks after a single insemination

(Reinhardt & Ribou, 2013), the average insemination rates of female bed bugs in semi-natural settings is approximately once per day (Yan & Dukas, 2022). This suggests sexual conflict over insemination rates, which we assessed in three experiments designed to quantify the fitness consequences of low and high insemination rates, and the behaviours of each sex that lead to the high insemination rates observed under semi-natural settings.

First, we compared the longevity, egg production rates, egg viability, and offspring production rates of females under low and high traumatic insemination rates informed by our data from semi-natural settings (Yan & Dukas, 2022). Importantly, our experimental design differed from prior studies (Morrow & Arnqvist, 2003; Stutt & Siva-Jothy, 2001) in that females were only briefly exposed to males each day under controlled settings, where we visually confirmed the occurrence of each insemination. This approach allowed us to minimize sexual harassment received by females. Sexual harassment involves relentless male pursuit of females, mountings, and sometimes coercive matings, which reduce female fitness (Dukas & Jongsma, 2012; Partridge & Fowler, 1990; Rice et al., 2006; Saveer et al., 2021). Given the potential energetic costs of wound healing and risks of infection associated with traumatic insemination in bed bugs (Reinhardt et al., 2003; Stutt & Siva-Jothy, 2001), along with evidence from three meta-analyses suggesting a negative association between mating rate and longevity (Arnqvist & Nilsson, 2000; Slatyer et al., 2012; South & Lewis, 2011), we predicted that females inseminated at higher rates would experience a reduction in lifespan. We also predicted that higher rates of traumatic insemination would be associated with decreased rates of egg production since mating has been shown to stimulate more rapid reproductive senescence in other species (Bretman & Fricke, 2019a; Priest et al., 2008). In combination, we predicted that these negative effects of high insemination rates on longevity coupled with a reduction in reproductive rates would result in overall decreased lifetime reproductive success in females inseminated at high vs. low rates.

Second, we experimentally manipulated female insemination status in order to critically test the effect of female insemination recency on male mountings, female avoidance, male rejection of females and traumatic insemination rates in realistic social

network settings. Taking a social-network approach, we assessed the effect of females' insemination status by observing replicate groups of 12 female and 12 male bed bugs in a large, semi-naturalistic arena where half of the females were manipulated to be recently inseminated (just before the test) while the other half were distantly inseminated (two days prior to the test). This approach blended elements of controlled laboratory and ecologically relevant field studies to provide insights into the interplay between male pursuit and female avoidance strategies in a dynamic group environment. While social network analyses have predominately been used to quantify social relationships (Farine & Whitehead, 2015; Pinter-Wollman et al., 2014; Wey et al., 2008), recent research has illustrated their utility for quantifying sexual interactions at the realistic level of social groups (Fisher et al., 2016; Greenway et al., 2021; McDonald et al., 2017; McDonald & Pizzari, 2017; Sih et al., 2009). We predicted that distantly and recently inseminated females would be mounted at equal rates because previous observations suggested that male bed bugs indiscriminately mount all bed bug-sized objects (Reinhardt & Siva-Jothy, 2007; Rivnay, 1933). We also predicted that, owing to the cost of high insemination rates, recently inseminated females would be more likely to avoid mounting males and hence be inseminated at lower rates than distantly inseminated females. Nonetheless, we also examined how often males aborted mounts directed at recently vs. distantly inseminated females to account for the possibility of male mate choice (Bonduriansky, 2001; Byrne & Rice, 2006; Edward & Chapman, 2011; Sargent et al., 1986).

Finally, we measured female avoidance under controlled settings, where females experienced successive daily inseminations over six days. We predicted that, as females receive an increasing number of inseminations, they would become progressively more resistant to male pursuit and insemination attempts, leading to longer insemination latencies and shorter insemination durations.

3.3 METHODS

3.3.1 Study population and maintenance

We used descendants of bed bugs (*Cimex lectularius*) collected from four sites in Southern Ontario between October 2019 and January 2020. We maintained the colony in two large 54 x 40 x 40 cm plastic storage bins kept at 27 ± 0.5°C and 60% relative humidity with lights off at 8:00 AM and on at 4:00 PM. This reversed lighting schedule allowed us to conduct our experiments during the dark phase, when bed bugs are active. Within the plastic bins, we housed bed bugs in 85 mL spice jars each containing several strips of folded filter paper to provide a rough surface for walking and oviposition. Each jar contained roughly 50 to 150 bed bugs of the same life stage. We fed the colony weekly under red light with defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using a Hemotek membrane-feeding system (Discovery Workshops, Accrington, UK). In all experiments, we generated virgin bed bugs by individually isolating recently fed fifth instar bed bugs and grouping them into same-sex groups once they emerged as adults.

3.3.2 The cost of traumatic inseminations

To quantify the cost of repeated traumatic inseminations, we compared the lifetime reproductive output of female bed bugs that were either inseminated daily or weekly. We selected one insemination per day as our high rate based on previously observed rates of traumatic insemination in bed bugs (Johnson, 1941; Stutt & Siva-Jothy, 2001; Yan & Dukas, 2022). Most notably, (Yan & Dukas, 2022) observed bed bugs in a complex, semi-naturalistic environment in which females had ample room and protective crevices to avoid excessive male pursuit and access to blood meals every other day. In this setting, females had a high average insemination rate of 0.89 ± 0.06 (mean ± SE) per day. As for our low-insemination-rate treatment, female bed bugs have been shown to continuously lay fertile eggs for up to 10 weeks after a single insemination (Reinhardt & Ribou, 2013), and thus once per week reflects a relatively low but likely sufficient rate of insemination. We first randomly assigned 20 one-week old, virgin adult females into each treatment. We housed each female in a 35 mm petri dish arena lined with filter paper and containing a dark shelter

tent folded from a 1 cm x 1 cm square of blue construction paper. We fed all females from both treatments six days before they were inseminated for the first time. The next day and every following week for the remainder of the experiment, we fed all females by briefly grouping them by treatment. We chose to feed weekly as bed bugs feed every 6-7 days when provided ad libitum access to blood (Stutt & Siva-Jothy, 2001). Twice a week, we moved each female into a fresh arena and then counted the number of eggs present in each recently occupied arena. We kept the old arenas with eggs for an additional eight days and then counted the number of first instar nymphs produced by each female. Counting of both eggs and offspring was conducted by observers blind to female treatment.

Every day for the high-insemination-rate females and once a week for the low-insemination-rate females, we conducted controlled insemination trials by placing a single male bed bug that had not mated for at least 48 hours into each arena and continuously inspected arenas to confirm that insemination occurred. We removed males immediately after they dismounted females to prevent additional inseminations. If insemination did not occur within ten minutes, we added a second male to each arena without removing the first male. We needed a second male for 6.8% of insemination trials and once an insemination began, we immediately removed the excess male to minimize additional interactions. On days where the low-insemination-rate females were not to be inseminated, we introduced a single male that had not mated for at least 48 hours with its copulatory organ superglued against its body to each arena. We then continuously observed these trials to ensure each female was mounted and pursued by males for approximately two minutes and to ensure that insemination did not occur. Note that, while we equalized male harassment of females between the treatments, we also minimized the harassment to a few minutes per day because such harassment decreases female bed bug fitness (Saveer et al., 2021). This allowed us to isolate the cost of traumatic insemination. On two occasions, we accidentally removed and discarded the focal female during insemination trials, resulting in a total sample size of 19 per treatment.

We ran all of our analyses using R v.4.1.1 (R Core Team, 2021). To compare survivorship between the high- and low-insemination-rate females, we fit a Cox

proportional hazard model using the *coxph* function from the *survival* package with days survived as the dependent factor and treatment as the independent factor (Cox, 1972; Therneau, 2022). Since we ended the experiment on day 77, when the final high-insemination-rate female died, the remaining 13/19 females from the low treatment were right-censored in the survival analysis. We analyzed differences in egg production of living females using a Generalized Linear Mixed Model (GLMM) with a negative binomial distribution where the response variable was number of eggs each individual female laid during the week. The model included an offset with the log of number of days during the week where each female was alive to account for occasions where females died mid-week and thus had fewer than seven days to lay eggs. If females were dead for the entire week, egg production was entered as NA. We included female treatment and week as fixed factors and arena number as a random factor. We next compared offspring production between females from each treatment by fitting a Linear Mixed Model (LMM) with treatment and week as fixed factors and arena number as a random factor. Here we wished to capture differences in female fitness, which encompasses longevity, and thus entered offspring production as zero for females even after they had died. Lastly, we analyzed differences in egg hatch rates using a GLMM with a binomial distribution and proportion of viable eggs per female per week represented using the *cbind()* function in R to combine hatched and unhatched eggs as the dependent variable. Once again, treatment and week were included as fixed factors and arena number as a random factor. For all models, we verified fits by inspecting plots of model residuals.

3.3.3 Effects of female insemination status on female avoidance and male rejection

Here we experimentally manipulated female insemination status in order to quantify female and male behaviours that lead to the high insemination rates observed under semi-natural settings. We observed five replicate groups of 12 male and 12 female bed bugs, where half of the females in each group were experimentally manipulated to be distantly inseminated and the other half recently inseminated. The distantly inseminated females were inseminated two days prior to the test while the recently inseminated females were

inseminated within 30 minutes of the start of the test phase. We first generated focal male and female virgin bed bugs that were each given a unique ID using paint from Sharpie oil-based paint markers after brief anesthetization with CO_2. We marked the bed bugs one week after they emerged as adults and began the first round of inseminations three days after marking.

To conduct controlled insemination trials, we individually placed focal female bed bugs into 35 mm petri dishes lined with filter paper. We then introduced a single non-focal virgin male bed bug into each petri dish while an observer ensured that insemination only occurred once. We first allowed non-focal males to inseminate all the 12 focal females. On the following day, we fed both male and female focal individuals. One day later, 30 minutes before the observation phase of the experiment, we generated the recently inseminated focal females by allowing a new set of non-focal males to inseminate six randomly selected focal females. While the recently inseminated females received a total of two inseminations compared to only a single insemination in the distantly inseminated females, these treatments reflected our semi-natural settings, in which females received about one insemination per day. We also allowed each of the 12 focal males to inseminate a single non-focal female to partially deplete their sperm and seminal fluid reserves as a means of promoting male choosiness during observations and to better reflect the natural conditions where males are unlikely to be virgin. A single insemination in male bed bugs depletes approximately 12% of their sperm and 19% of their seminal fluid volume (Reinhardt et al., 2011). For all insemination trials, when insemination did not occur within 10 minutes, we added another virgin non-focal bed bug of the opposite sex to the arena to ensure insemination.

Once all insemination trials were completed, we immediately placed the 12 mated males, 6 recently inseminated, and 6 distantly inseminated females into a 34.5 x 23.5 x 15 cm Plexiglass experimental arena lined with filter paper (Fig. 3.1a). In the arena, we placed six wooden shelters constructed from balsa wood slat segments covered with glass microscope slides (Fig. 3.1a). Each of these shelters are sufficiently spacious to accommodate all 24 adult bed bugs. We then documented all sexual interactions along with

which individuals were involved in each interaction and the outcome of each interaction through continuous observation for one hour while ensuring observers were blind to female treatment. The flowchart in Figure 1b illustrates how sexual interactions and outcomes were scored. We recorded all mounts directed at females followed by whether the female attempted to avoid the mount. Attempted avoidance involved either running away or displaying the refusal postures described by Siva-Jothy (Siva-Jothy, 2006). If females did not avoid or failed to avoid a mount, we then recorded whether males aborted or proceeded with insemination. Inseminations were characterized by males remaining securely mounted with their abdomen curled underneath a female's right abdomen for longer than 20 seconds (Carayon, 1966). Mounts, on the other hand, appear as a male quickly "jumping" onto a female (Stutt & Siva-Jothy, 2001). Both traumatic insemination and mounting are highly stereotyped and distinctive behaviours. Additionally, we validated our insemination criteria in a prior study where 92% of once-inseminated females produced offspring while no females in a non-inseminated reference group laid eggs (Yan & Dukas, 2022).

We analyzed female avoidance rate, male rejection rate, and insemination rate as a function of female insemination status by constructing three generalized linear mixed-effects models (GLMMs) in R using the package *lme4* (Bates et al., 2015). We verified model fits by visually inspecting plots of model residuals using the *DHARMa* package (Hartig, 2019) and report Wald χ^2 values derived from the *Anova* function from the *car* package (Fox et al., 2012). We fit binomial logistic regressions for all three models and included treatment as a fixed factor and replicate, male ID, and female ID as random factors. Each of these three models had binary outcomes of mounts as the response variable. The female avoidance rate model assessed whether females attempted to avoid each mount directed at them. The male rejection rate model assessed whether males aborted each mount where they had the option to abort. This excluded mounts that females successfully avoided. Finally, the insemination rate model assessed whether mounts resulted in insemination. We predicted that recent insemination would be associated with higher female avoidance rates, higher male rejection rates and, consequently, lower insemination rates.

We created all network visualizations with R v.4.1.1 (R Core Team, 2021) using the *igraph* package (Csardi & Nepusz, 2006). For each of the five replicates, we created one mount and one insemination network. Mount networks display all mounts, including ones that eventually resulted in insemination. Each node represents an individual bed bug and node colours denote sex and treatment. In mount networks, weighted edges correspond to number of mounts that occurred between two individuals. In insemination networks, weighted edges correspond to the number of inseminations that occurred between two individuals. Directed edges indicate who initiated and who was the recipient of each sexual interaction. While males do mount other male bed bugs (Rivnay, 1933; Ryne, 2009), we ignored these interactions during observations to ensure that we accurately captured all male-female interactions as this was the focus of our study. Therefore, all edges in our sexual networks go from males to females. Lastly, node size corresponds to network strength (weighted degree), which is equivalent to the sum of all edge weights connected to a node. For example, larger female nodes in a given mount network represent females that received more mounts compared to smaller female nodes in the same network. We gave each individual within each replicate a unique letter ID and held node position constant between mount and insemination networks within the same replicate. These networks allow us to visualize which females were mounted and inseminated more based on overall differences in node size and connectedness (number of edges directed at each female). We did not test whether differences in network metrics were statistically significant as the analyses above already addressed our study questions and further analyzes would have been redundant.

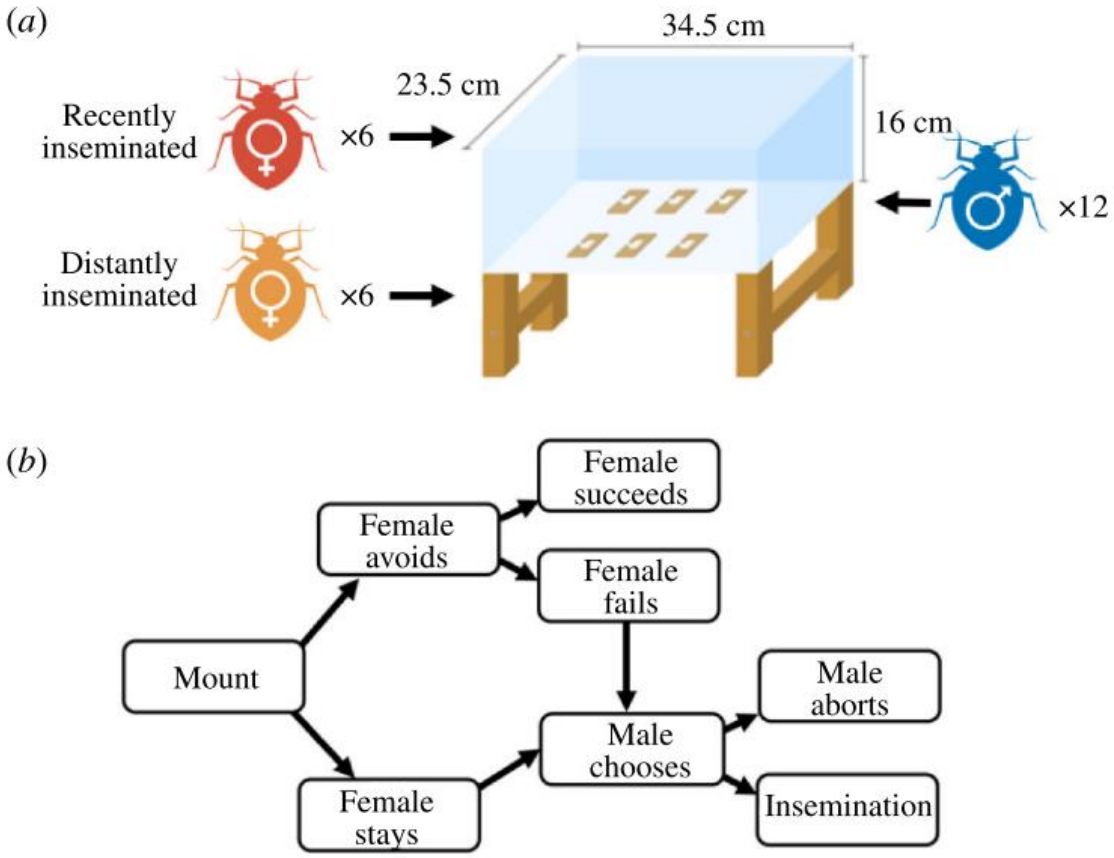

Figure 3.1. (a) Schematic overview of the experimental design and arena set-up including the positions of shelters. We observed interactions between six recently inseminated females, six distantly inseminated females and 12 standard males per replicate. (b) Flowchart illustrating how we scored sexual interactions and their outcomes.

3.3.4 Effect of repeated traumatic inseminations on female avoidance

Here we wished to test whether focal females show greater avoidance of males after receiving inseminations over 6 successive days. We video-recorded insemination trials for 13 focal females as they got inseminated once daily for six consecutive days. All females emerged as adults one week before the start of the experiment. We also ensured that all females fed one day before the first day of the insemination trials. Each day, we placed each of the 13 females in a 35 mm petri dish arena lined with filter paper and added a same-age male that had not mated for at least 48 hours. Trials lasted until insemination ended or once 20 minutes had elapsed since the male was added. There were only two instances where focal females were not inseminated within the 20-minute trial. For these instances, insemination latencies and duration were entered as NA while female avoidance rate was calculated as usual with trial duration lasting the full 20 minutes. One instance of a focal female not getting inseminated happened on the last day of recordings, so we did not need to ensure that the female was inseminated after the trial ended. In the other instance, we added a new male to the unmated female's arena and ensured that insemination occurred so that we could continue using the female for the rest of the experiment. Between the daily insemination trials, we housed the focal females in an 85 mL jar containing folded strips of filter paper.

To video record trials, we used eight 6th generation iPod Touches that captured two arenas at a time. Then, using BORIS observation software (Friard & Gamba, 2016) to score the videos we recorded, an observer blind to both the treatment and day of each trial recorded the time of first encounter, the amount of time females spent either running away from males or in the refusal posture, and the start and end times of insemination, allowing us to calculate female avoidance rate, insemination latency, and insemination duration. Every day, we also simultaneously recorded insemination trials for a new set of 13 virgin females that served as a reference group allowing us to control for day effects. These reference trials using virgin females allowed us to obtain baseline measures of insemination latencies, insemination durations, and avoidance rates that account for day-to-day fluctuations in weather or environmental variables that have been shown to influence insect

behaviour, even in controlled laboratory settings (Austin et al., 2014; Roitberg et al., 1993). For each of our three response variables, we controlled for day effects by subtracting the daily mean of our virgin reference females' scores from each focal female's avoidance rate, insemination latency, or insemination duration score. Because this subtraction occasionally resulted in negative values, we added a positive integer constant to each female score, allowing us to log-transform response variables to meet model assumptions. To calculate female avoidance rate, we looked at the proportion of time between first encounter and the start of insemination that a female spent either running away or in the refusal posture. This window of time captured the portion of the trial when males were pursuing females. Next, we fit a LMM with the log of avoidance rate as the response variable, number of prior inseminations as a fixed effect, and arena number as a random factor. Insemination latency was based on the time it took from first encounter to the start of insemination. We fit a LMM with the log of insemination latency as the response variable, number of prior inseminations as a fixed effect, and arena number as a random factor. Lastly, we fit a LMM with insemination duration as the response variable, number of prior inseminations as a fixed factor, and arena number as a random factor. For all models, we verified fits by inspecting plots of model residuals.

3.4 RESULTS

3.4.1 The cost of traumatic insemination

The high-insemination-rate females had drastically lower survivorship than the low-insemination rate-females (Cox regression: Wald $\chi^2_1 = 18.85$, $p < 0.0001$; Fig. 3.2a). In fact, by the time all high-insemination-rate females had died, 68% of the low-insemination-rate females were still alive. Furthermore, the low-insemination-rate females produced eggs at a higher rate compared to high-insemination-rate females (GLMM: Wald $\chi^2_1 = 28.03$, $p < 0.0001$; Fig. 3.2b). Overall, the observed differences in longevity and egg production rate resulted in the low-insemination-rate females producing significantly more offspring than the high-insemination-rate females (LMM: Wald $\chi^2_1 = 42.95$, $p < 0.0001$; Fig. 3.2c). As we terminated the experiment before a majority of low-insemination-rate females had died, our

results represent a lower estimate of the true cost of repeated traumatic inseminations. Lastly, we found that hatch rate was non-significantly, 0.9% higher in the low- than high-insemination-rate treatments (Mean ± SE for proportion of eggs hatched: Low = 0.98 ± 0.003, High = 0.97 ± 0.005; GLMM: Wald $\chi^2_1 = 2.0$, $p = 0.16$).

3.4.2 Effects of female insemination status on female avoidance and male rejection

A greater proportion of mounts directed at distantly inseminated females resulted in inseminations (GLMM: Wald $\chi^2_1 = 7.572$, $p < 0.01$; Figs 3.3a, b, and c, Fig. S3.1). This pattern was not driven by female avoidance behaviour as we did not detect any differences in the propensity to avoid mounts between females of the two treatments (GLMM: Wald $\chi^2_1 = 0.99$, $p = 0.32$; Fig 3.3d). Males, however, were much more likely to abort insemination attempts with recently than with distantly inseminated females (GLMM: Wald $\chi^2_1 = 6.43$, $p < 0.05$; Fig 3.3e).

3.4.3 Effect of repeated traumatic inseminations on female avoidance

Female avoidance rates increased as a function of the number of prior inseminations (LMM: Wald $\chi^2_1 = 5.35$, $p < 0.05$; Fig 3.4a). This was driven by a marked increase in avoidance behaviour following the females' third consecutive insemination. However, increases in avoidance behaviour did not result in increased insemination latency over the course of the experiment (LMM: Wald $\chi^2_1 = 1.12$, $p = 0.29$; Fig 3.4b). Lastly, as females received a greater number of inseminations, the duration of each insemination decreased (LMM: Wald $\chi^2_1 = 6.65$, $p < 0.01$; Fig 3.4c).

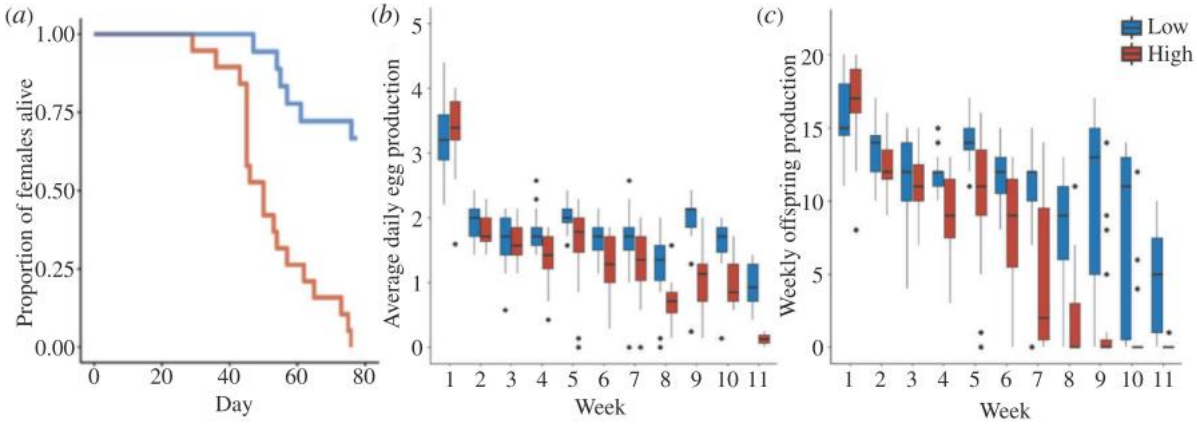

Figure 3.2. (a) Survival curves for females in the low, weekly insemination rate treatment group (blue; n = 19) and high, daily insemination rate treatment group (red; n = 19). (b) Average daily egg production rates for living females from the low- and high-insemination-rate groups. The initial sample sizes for each treatment are 19, but they gradually decrease with female death, culminating in 14 and 2 for the low- and high-insemination-rate groups, respectively. Bold horizontal lines indicate the medians, the boxes represent the IQR between the first and third quartiles, and the whiskers above and below each box represent values within ±1.5 of the IQR. Diamonds indicate outliers. (c) Weekly offspring production for females in the low- (blue) versus high- (red) insemination-rate groups. Note that this panel includes all females, both alive and dead (n = 19 females for each treatment).

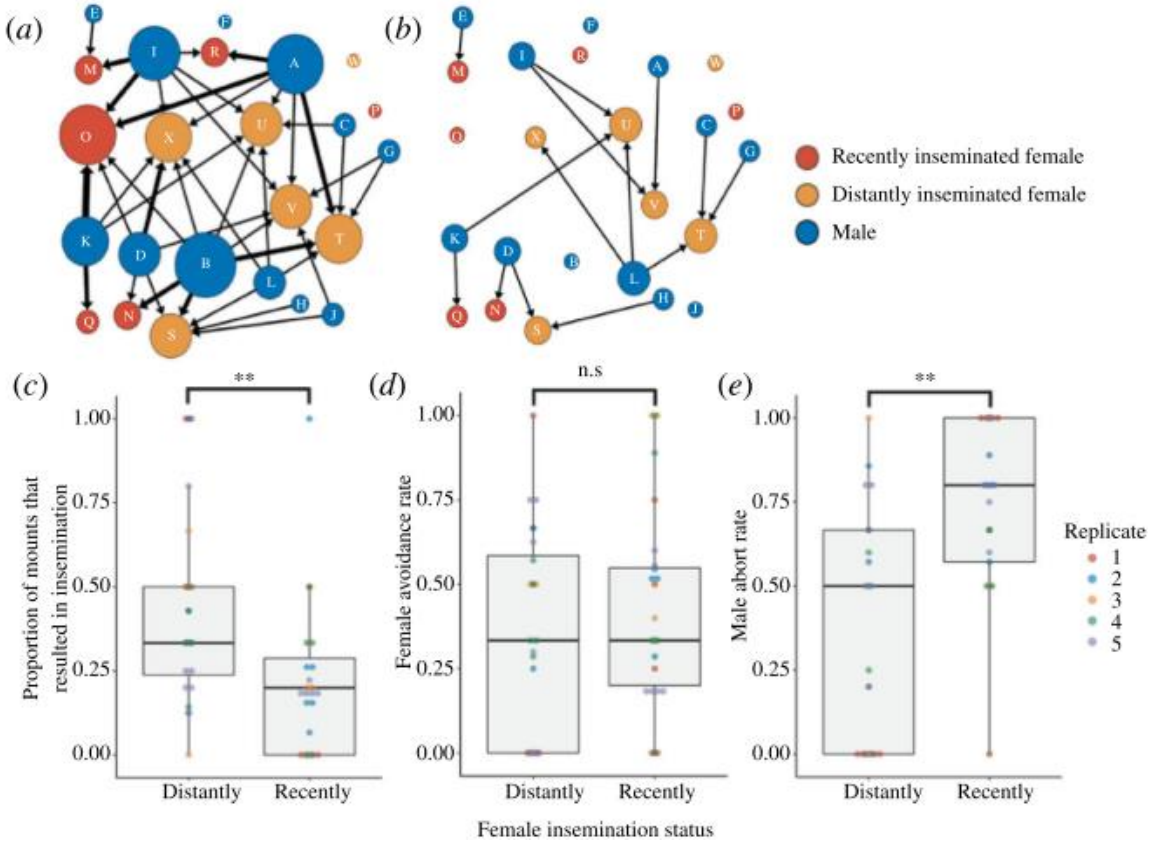

Figure 3.3. Weighted and directed sexual networks from a sample replicate depicting (a) mounts and (b) inseminations. Each node represents an individual bed bug and is labelled with a letter ID. The position of each individual is held constant in the two networks. Node colour depicts treatment and sex as indicated in the legend, while node size and edge width increase with strength (weighted degree). Sexual networks for all replicates are depicted in electronic supplementary material, figure S1. (c) Insemination rate, (d) avoidance rate and (e) male abort rate for distantly (n = 30) versus recently (n = 30) inseminated females. Each boxplot shows the raw values for each individual female and differently coloured data points refer to females from different replicates as indicated by the legend. Electronic supplementary material, figure S2 consists of boxplots depicting the total number of mounts and inseminations received by each female.

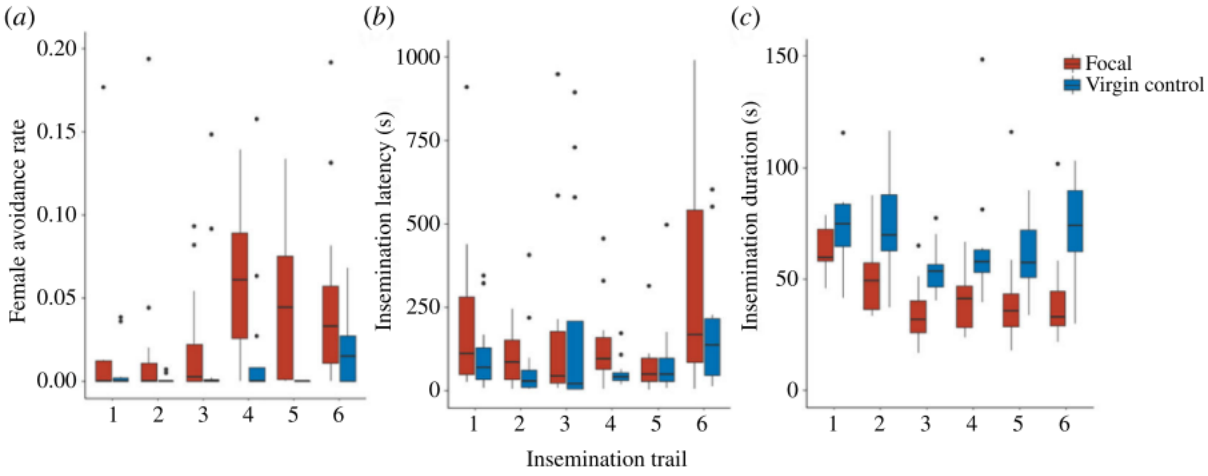

Figure 3.4. (a) Female avoidance rate, measured as the proportion of trial duration spent avoiding males, (b) insemination latency and (c) insemination duration for focal females that were inseminated once daily for six consecutive days (red; n = 13 females total, except for days 1 and 6 of the insemination latency and duration figures where n = 12, because one of the 13 females was not inseminated during the trial) and virgin reference females (blue; n = 13 females per day). The same group of 13 focal females were tested while a new group of age-matched virgin control females were used each day to control for day effects.

3.5 DISCUSSION

Through tracking females that were inseminated at either daily rates or weekly rates, we found that daily rates of traumatic insemination resulted in a dramatic reduction in fitness. Females subjected to daily inseminations experienced reduced longevity, egg production, and offspring production (Fig. 3.2). Next, we created replicate social networks of 12 male and 12 female bed bugs, where half of the females were recently inseminated while the other half were distantly inseminated. We found that a fewer proportion of mounts directed at recently inseminated females resulted in subsequent inseminations. However, to our surprise, recently inseminated females did not avoid males more frequently than distantly inseminated females. Instead, the observed difference in insemination rate was driven by males rejecting recently inseminated females at higher rates than they rejected distantly inseminated females (Fig. 3.3). To resolve the apparent contradiction between the high cost of multiple inseminations in our first experiment and lack of difference in female avoidance rates in the second experiment, we tracked female avoidance behaviour under controlled settings and found that females displayed more avoidance behaviour as they received more inseminations (Fig. 3.4). This increase in avoidance behaviour, however, did not result in longer insemination latencies (Fig. 3.4). Lastly, insemination duration decreased over time (Fig. 3.4).

In the past few decades, the study of polyandry has received increasing scientific attention with an emphasis on the various potential fitness benefits and costs to females from mating with multiple males. The accumulation of studies on polyandry has demonstrated that a single mating typically does not maximize females' fitness, with two meta-analyses reporting net fitness gains as high as 30-70% as a consequence of multiple mating (Arnqvist & Nilsson, 2000; South & Lewis, 2011). In our current study, however, we found that high, daily rates of traumatic insemination dramatically reduced survivorship and lifetime offspring production (Fig. 2a, b, c). Since most of our low-insemination-rate females were still alive when we terminated the experiment, the true cost of daily insemination it likely higher than our result. Studies on multiple species of crickets, flies, and beetles, however, have shown that polyandry can elevate female indirect fitness

through increased egg hatch success rates (Baker et al., 2001; Simmons, 2005; Tregenza & Wedell, 1998; Worden & Parker, 2001). Yet, our data also did not support indirect (genetic) benefits as we did not detect any differences in egg hatch rate between the low and high-insemination-rate females. It remains possible that high-insemination-rate females benefitted from other indirect benefits that we did not quantify, such as increased offspring quality. However, such genetic benefits are unlikely to offset the >50% reduction in direct fitness we reported. Moreover, there is currently limited evidence that female multiple matings improve offspring performance metrics in other taxa (Simmons, 2005). Nonetheless, future studies should consider measuring offspring traits to examine potential indirect fitness benefits of high mating rates.

Overall, our results suggest that, while polyandry has generally been reported to be beneficial to females (Arnqvist & Nilsson, 2000; Slatyer et al., 2012; Snook, 2014; South & Lewis, 2011), some instances of polyandry may arise from sexual coercion and sexual conflict over mating rate, thus resulting in net fitness costs for females. For example, multiple mating has been shown to be costly in multiple species of beetles (den Hollander & Gwynne, 2009; Okada et al., 2017a; Orsetti & Rutowski, 2003; Rönn et al., 2006; Sakurai & Kasuya, 2008), fruit flies (Bretman & Fricke, 2019b; Fowler & Partridge, 1989; Priest et al., 2008), and water striders (Arnqvist, 1989a). It is currently unclear as to whether the variation in reported consequences of polyandry across studies reflects taxonomic differences or discrepancies in the mating rates and experimental conditions employed across studies. A key distinction between our study and most existing studies assessing the consequences of polyandry is that we exposed females to different mating rates throughout their lifetime until all females of one treatment died. Currently, estimates of the costs and benefits associated with multiple mating overwhelmingly come from experiments that compare only one vs. two or one vs. a small handful of matings, which likely do not capture realistic rates of female multiple mating in most species. In fact, only examining the small handful of studies that test higher rates of multiple mating reveals that higher rates of polyandry are often either not beneficial (South & Lewis, 2011), or even detrimental (Arnqvist & Nilsson, 2000), to female fitness. This pattern has led researchers to suggest

that females may experience an optimal intermediate mating rate, where further elevated rates of mating become deleterious (Arnqvist & Nilsson, 2000). Our results add to the small body of literature that test such elevated mating rates and demonstrate that high rates of polyandry can indeed be costly to females. To better understand the trade-offs associated with polyandry and critically test if females exhibit an optimal intermediate mating rate, future studies should examine female fitness under a broader range of mating rates that ideally capture what females in each species would naturally experience.

It is important to note that our results from Experiment 1 (Fig. 3.2) fall in line with Stutt and Siva-Jothy's (Stutt & Siva-Jothy, 2001) but only partially with Morrow and Arnqvist's (Morrow & Arnqvist, 2003) experiments on multiple traumatic inseminations in bed bugs. Both our study and Stutt and Siva-Jothy (Stutt & Siva-Jothy, 2001) found that high rates of insemination reduce female fitness while Morrow and Arnqvist (Morrow & Arnqvist, 2003) found a decrease in female survivorship but no overall differences in fitness for females inseminated at high vs. low rates. This discrepancy can be attributed to the fact that we controlled for and minimized the effect of sexual harassment by exposing females to males for very short durations. Both prior studies continuously housed males with females, thereby exposing females to continuous harassment. Additionally, while both studies attempted to equalize harassment levels using males with glued genitals, we observed that males with glued genitals pursued females more relentlessly than control males (J.L.Y., M.L.D., personal observations). Therefore, harassment levels were likely highly variable between studies and between treatments in Stutt and Siva-Jothy (Stutt & Siva-Jothy, 2001) and Morrow and Arnqvist's (Morrow & Arnqvist, 2003) experiments. Sexual harassment has been shown to reduce elements of female fitness in a wide array of species (Helinski & Harrington, 2012; Okada et al., 2017b; Partridge & Fowler, 1990; Réale et al., 1996; Rice et al., 2006; Sakurai & Kasuya, 2008), including bed bugs (Saveer et al., 2021). Therefore, to minimize inconsistencies between studies, increase replicability, and better isolate the effect of repeated matings, we suggest that future studies evaluating the fitness consequences of polyandry across taxa adopt protocols that both control for and reduce sexual harassment of females.

In our second experiment, which was conducted in a semi-naturalistic arena (Fig. 3.1a), we found that females' insemination recency affected the frequency at which subsequent mounts directed at them resulted in inseminations (Fig. 3.3c). This is illustrated in the social networks (Fig. 3.3a, b, Fig. S3.1), which depict similar node sizes and connectivity of females from both treatments in the mount networks, but larger and more connected nodes of distantly inseminated females in the insemination networks. While we predicted that the high cost of multiple inseminations would drive recently inseminated females to avoid mounts more frequently, we found no difference between the treatments (Fig. 3.3d). In this experiment, however, the recently inseminated females received only two inseminations prior to the test. We later found in our third experiment that females increased avoidance behaviour only following three successive daily inseminations (Fig. 3.4a). Hence, we likely required a higher number of successive inseminations in the second experiment to observe increased female avoidance rates. Alternatively, it is possible that running away is not an effective strategy. Nonetheless, given the immense amount of variation in females' propensity to avoid mounts (Fig. 3.3d), it would be worthwhile to investigate other factors that may predict avoidance behaviour including female body condition and individual experience such as the outcomes of previous avoidance attempts. For instance, long-term exposure to males has been shown to increase swimming performance and aerobic capacity to facilitate escape from males in female Trinidadian guppies (*Poecilia reticulata*) (Killen et al., 2016).

We found that males aborted mounts more frequently with recently than distantly inseminated females (Fig. 3.3e). These results show that males respond to perceived sperm competition by foregoing insemination opportunities entirely, thus representing a clear example of male mate choice. Examples of male mate choice have been reported in an increasing number of species across, insects, fishes, birds, and mammals (Bonduriansky, 2001; Byrne & Rice, 2006; Jones et al., 2001; Reading & Backwell, 2007; Sargent et al., 1986; Schwagmeyer & Parker, 1990), and challenge the assumption from classical sexual selection theory that males should mate indiscriminately and at every given opportunity (Bateman, 1948; Trivers, 1972). It is possible that the males in our study expected to

encounter virgin or non-recently inseminated females in the near future and therefore conserved their limited sperm and seminal fluid reserves (Reinhardt et al., 2011). However, many additional factors like physiological limitations, sperm precedence patterns, and variation in female quality can play a role in how males exhibit mate choice and respond to sperm competition. How these different factors interact remain poorly understood (Parker & Pizzari, 2010; Wedell et al., 2002).

In our third experiment, we found that females increased the proportion of time spent avoiding males as they received six successive daily inseminations, with a notable increase on the fourth day, after females had already received three prior inseminations (Fig. 3.4a). First, this provides evidence that females possess plastic behavioural avoidance strategies based on their own sexual history, which may help mitigate the costs of repeated inseminations. Furthermore, our observation that females spent nearly no time avoiding males until the fourth daily insemination session suggests that up to three inseminations either increase or do not decrease their fitness. Across taxa, females may benefit from a small number of matings as opposed to one to protect against mating failure (Greenway & Shuker, 2015; Tyler & Tregenza, 2013), increase the genetic diversity of their offspring (Garcia-Gonzalez et al., 2015; Yasui, 1998; Yasui & Garcia-Gonzalez, 2016), or enhance fecundity through receiving more beneficial ejaculate components (Arnqvist & Nilsson, 2000; Reinhardt et al., 2009a; South & Lewis, 2011). Despite females spending more time avoiding males following their third consecutive insemination, insemination latency did not increase over successive daily trials (Fig. 3.4b). This was likely an artifact caused by our use in this experiment of the small 35 mm arenas. Such limited space was necessary for quantifying subtle behaviours via close-up video recording, but limited females' abilities to avoid males.

Lastly, we replicated previous findings showing that a female's first insemination lasts significantly longer than subsequent inseminations (Fig. 3.4c) (Siva-Jothy & Stutt, 2003). While we cannot rule out the possibility of females influencing males to terminate insemination, we have rarely observed changes in female behaviour that resulted in the terminations of inseminations. Therefore, seeing as insemination duration is positively

correlated with the amount of ejaculate transferred (Reinhardt et al., 2011), males appear to be investing more heavily in virgin females possibly because the absence of rival sperm signals lower sperm competition. This is consistent with our results from Experiment 2, where males displayed a preference for distantly over recently inseminated females (Fig. 3.3e), and our previous findings, where males preferred the social cues of virgin vs. mated females (Yan & Dukas, 2022). However, currently, there is limited evidence suggesting weak last male sperm precedence in bed bugs (Stutt & Siva-Jothy, 2001). Yet, theoretical models predict greater investment in virgin females in mating systems with first rather than last male sperm precedence (Parker & Pizzari, 2010; Wedell et al., 2002). It is therefore evident that further research is required to uncover the patterns and mechanisms affecting sperm usage to understand the full scope of male and female sexual strategies under intense sperm competition.

In conclusion, we found strong evidence that high rates of one traumatic insemination per day in bed bugs result in dramatic fitness costs for females. Although females do increase their rate of avoiding sexual pursuit following multiple prior inseminations, why females endure insemination rates far above their apparent optimum remains unknown. Overall, these findings, coupled with our documentation of male mate choice, suggest that males predominantly control insemination rates in bed bugs and provide greater insight into how high rates of mating with multiple males can affect female fitness and male reproductive investment. Future work should focus on uncovering the direct and indirect fitness consequences of mating under a broader range of mating rates and examining the role that post-copulatory sexual selection mechanisms like sperm precedence patterns play in male and female sexual strategies.

3.6 ACKNOWLEDGEMENTS

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3.8 SUPPLEMENTARY INFORMATION

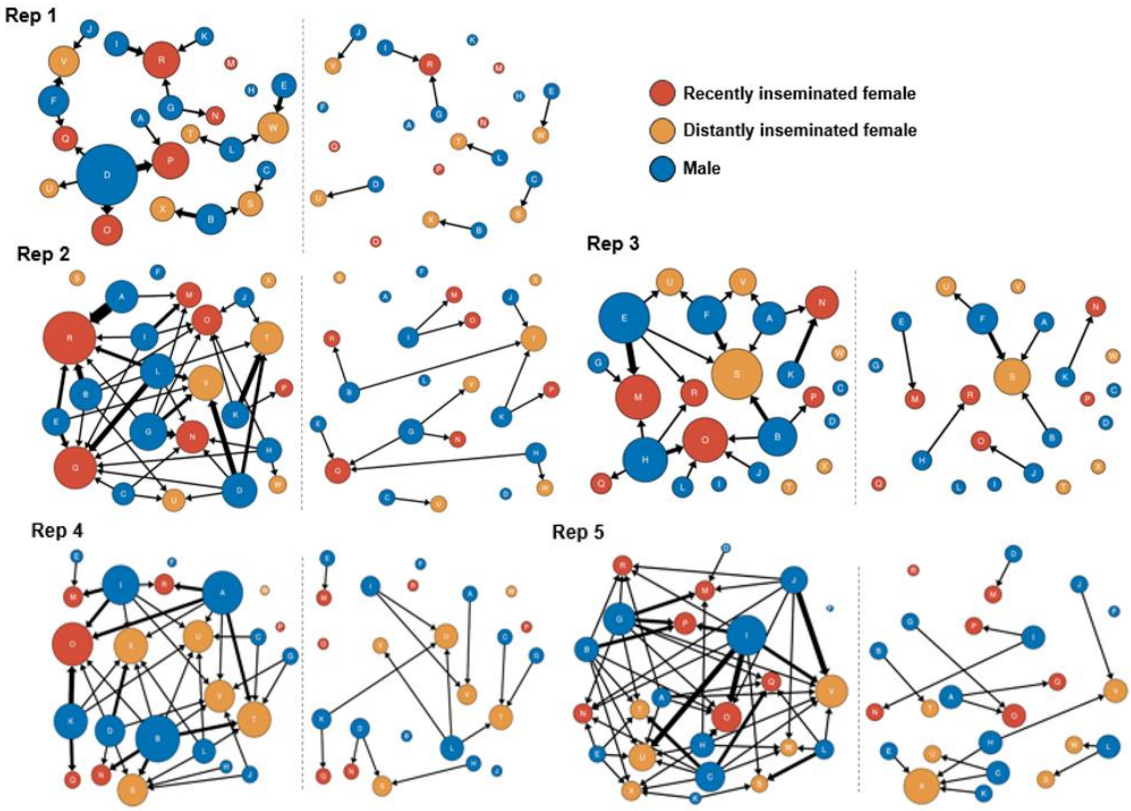

Figure S3.1. Weighted and directed sexual networks for all replicates with mounts networks on the left and insemination networks on the right. Each node represents an individual bed bug and is labelled with a letter ID. The position of each individual is also held constant between the two networks. Node colour depicts treatment and sex as indicated in the legend while node size and edge width increase with strength (weighted degree).

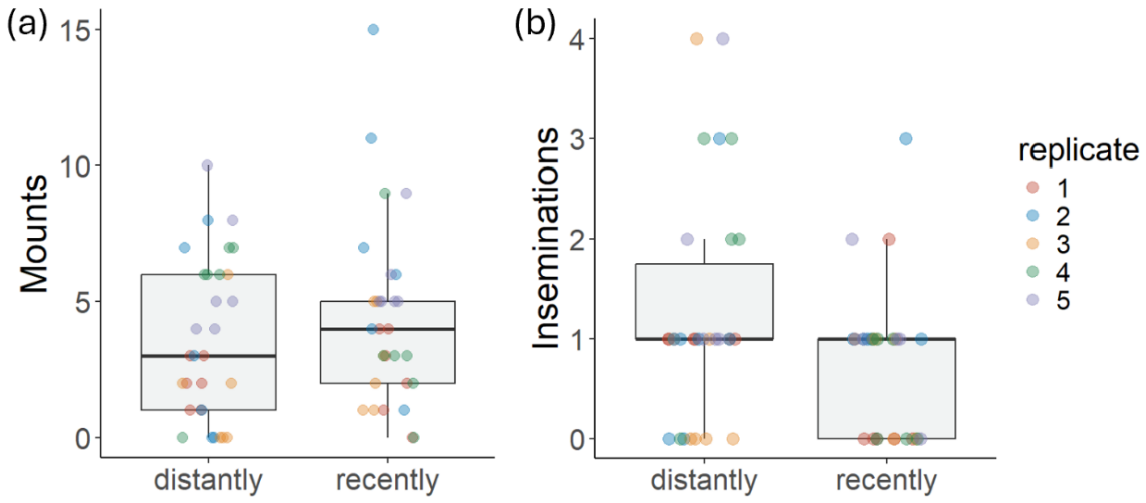

Figure S3.2. Total number of (a) mounts and (b) inseminations received by distantly (N = 30) vs. recently (N = 30) inseminated females. Each boxplot shows the raw values for each individual female and differently coloured data points refer to females from different replicates as indicated by the legend.

CHAPTER 4 – SEXUAL CONFLICT AND SOCIAL NETWORKS IN BED BUGS: EFFECTS OF SOCIAL EXPERIENCE

Yan, J.L., Rosenbaum, J.R.*, Esteves, S.*, Dobbin, M.L.*, Dukas, R. (2024). Sexual conflict in bed bugs: effects of social experience. Behavioral Ecology, 35(3): arae030.

4.1 ABSTRACT

Living in groups can provide essential experience that improves sexual performance and reproductive success. While the effects of social experience have drawn considerable scientific interest, commonly used behavioural assays often do not capture the dynamic nature of interactions within a social group. Here, we conducted three experiments using a social network framework to test whether social experience during early adulthood improves the sexual competence of bed bugs (*Cimex lectularius*) when placed in a complex and competitive group environment. In each experiment, we observed replicate groups of bed bugs comprising previously socialized and previously isolated individuals of the same sex, along with an equal number of standardized individuals of the opposite sex. Regardless of whether we controlled for their insemination history, previously isolated males mounted and inseminated females at significantly higher rates than previously socialized males. However, we found no evidence of social experience influencing our other measures of sexual competence: proportion of mounts directed at females, ability to overcome female resistance, and strength of opposite-sex social associations. We similarly did not detect effects of social experience on our female sexual competence metrics: propensity to avoid mounts, rate of successfully avoiding mounts, opposite-sex social association strength, and rate of receiving inseminations. Our findings indicate that early social experience does not improve sexual competence in male and female bed bugs.

4.2 INTRODUCTION

Social experience can drastically shape the physiology (Crews et al., 1997; Sachser & Lick, 1991), brain-structure (Champagne & Curley, 2005; Crews et al., 1997), cognitive abilities

(Chapman et al., 2008; Taborsky et al., 2012), and behaviour (Lihoreau et al., 2009; Polt & Hess, 1966) of animals. Experience with same-sex conspecifics can provide crucial information about local levels of competition, allowing for plastic responses that increase an individual's fitness (Bretman et al., 2010a; Gage & Baker, 1991). Experience with same-sex conspecifics can also be essential for learning skills related to courtship and mating. For example, male zebra finches (*Taeniopygia guttata*) require the presence of other adult males during development to adequately learn courtship singing and develop a preference for opposite-sex stimuli, two essential components of mating success in this species (Adkins-Regan & Krakauer, 2000; Immelmann, 1969).

Opposite-sex interactions can similarly be crucial as they provide opportunities for sexual experience leading to better performance in future sexual encounters (Milonas et al., 2011; Orihuela & Aguirre, 2011; Pérez-Staples et al., 2010). Even failed mating attempts can provide individuals with valuable feedback from unreceptive individuals, which can improve sexual pursuit strategies and aid in narrowing the range of sexual partners one pursues (Dukas, 2004, 2005). The overall importance of social experience is further exemplified by the fact that social isolation has been shown to alter an individual's subsequent behaviour and fitness in mammals (Harlow et al., 1965), birds (Baron & Kish, 1960), fishes (Hesse & Thünken, 2014; Taborsky et al., 2012), and insects (Baxter & Dukas, 2017).

Despite the well-documented effects of social isolation across taxa (Cacioppo & Hawkley, 2009; Gerall et al., 1967; Kim & Ehrman, 1998), prior studies that experimentally manipulate social experience largely assess its influence using simple mating and behavioural assays on either individual behaviour or dyadic interactions (Arnold & Taborsky, 2010; Favati et al., 2021; Guevara-Fiore, 2012; Lehtonen et al., 2016; Řežucha & Reichard, 2014; Sakata et al., 2002). While highly informative, these approaches do not reflect the full range of social and environmental pressures that most animals face under natural settings. For example, commonly used behavioural assays typically do not capture challenges associated with mate search, competition from rivals, and the possibility for females to successfully evade undesired sexual pursuit. Furthermore, while there has been

ample research on how experience shapes male courtship and mating strategies (Bailey et al., 2010; Dukas, 2005; Jordan & Brooks, 2012; Lehtonen et al., 2016; Řežucha & Reichard, 2014; Rodríguez et al., 2013), few studies have investigated the effects of social experience using a sexual conflict framework to examine whether prior experience influence females' tendency and ability to evade costly pursuit (but see Killen et al., 2016 for a notable exception). Yet, seeing as sexual conflict and male harassment of females is pervasive among sexually reproducing animals (Chapman, 2006; Clutton-Brock & Parker, 1995; Parker, 1979), we may expect females to exhibit behavioural plasticity in response to prior exposure to conspecifics. Overall, our current understanding of how social experience influences both males' and females' subsequent behaviours remains limited. Therefore, we sought to address fundamental knowledge gaps by examining how the social environment in early adulthood shapes the subsequent sexual competence of both males and females in a naturalistic, complex, and competitive environment using bed bugs (*Cimex lectularius*) as a model system.

Bed bugs are a frequently cited example of extreme sexual conflict as they obligately reproduce through traumatic insemination, whereby males pierce and deposit sperm directly into females' abdomens. Likely owing to the energetic costs of wound healing and increased frequency of infection, realistic rates of repeated traumatic insemination have been shown to dramatically reduce both longevity and lifetime reproductive output of female bed bugs (Stutt & Siva-Jothy, 2001; Yan et al., under review). Thus, while males should try to maximize number of inseminations, females are under selective pressure to evade excess inseminations. Bed bugs also display moderate social behaviour. They are typically found in mixed-sex aggregations within tight crevasses and possess volatile, non-volatile, and tactile cues that facilitate social attraction (Carayon, 1966; Reinhardt & Siva-Jothy, 2007; Siljander et al., 2007, 2008; Yan & Dukas, 2022). Combined, these sexual and social features of bed bugs make them an ideal model for examining how one's prior social environment can shape sexual competence, since group-living may lead to the acquisition of information and experience that can improve the outcome of subsequent sexual interactions.

In addition to documenting the individuals involved and outcomes of sexual interactions, we also constructed social networks based on hourly scans of bed bugs' social partners. Occupying more central network positions in mixed-sex (Formica et al., 2012; Oh & Badyaev, 2010; Ryder et al., 2008) and opposite-sex association networks (Beck et al., 2021; Dunning et al., 2023; Godfrey et al., 2012; Sabol et al., 2020) has been positively linked to mating success and fitness in several taxa. However, the traits and environmental factors that determine an individual's position in a social network remain poorly understood. There are currently only a small handful of studies assessing the relationship between social experience and future network position (Crailsheim et al., 2020; Kurvers et al., 2020; McDonald, 2007) and even fewer that experimentally manipulate experience to explicitly test its effects on various network metrics (Bentzur et al., 2021; Brandl et al., 2019; Riley et al., 2018). To address these gaps, we examined how prior experience with conspecifics influences the strength of individuals' opposite-sex associations. We focused on opposite-sex social associations as we believed they would represent a crucial aspect of bed bugs' social environments given that they exhibit extreme polyandry and sexual conflict. Since prior animal network studies have documented strong links between social network position and several measures of reproductive success (Beck et al., 2021; Dunning et al., 2023; McDonald et al., 2019a), we expected strong connections to opposite-sex conspecifics to reflect males' ability to locate females and retain continued access to insemination opportunities, and females' inability to successfully find refuge from continued sexual pursuit from males.

We first examined how social experience shapes the subsequent sexual competence of males by generating groups of previously isolated males, previously socialized males, and new, unfamiliar females, and observing all sexual and social interactions within complex arenas with multiple shelters. This approach created a dynamic setting where experienced and inexperienced males directly competed with one another for access to females in an environment where females could readily escape insemination attempts. We predicted that, compared to previously isolated males, socially experienced males would direct a higher proportion of mounts at females as opposed to males, be better at

overcoming female avoidance, form stronger opposite-sex social network associations, and achieve higher overall insemination rates. Second, we tested the effects of social experience on female bed bugs' sexual competence. Specifically, we examined whether previously socialized females were more adept at avoiding costly inseminations compared to previously isolated females. Since we expected females in the social treatment to gain relevant experience in avoiding persistent sexual pursuit in a complex environment with several males, we predicted that previously socialized females would attempt to evade more mounts, successfully evade more mounts, form weaker opposite-sex social network associations, and be inseminated at lower overall rates compared to previously isolated females.

4.3 METHODS

4.3.1 Ethical Note

Our research complied with all laws and did not require ethics committee approval. While we do not require ethics approval, we treat our subjects in accordance with strict animal ethics standards under the assumption that they experience emotion in general and pain in particular.

4.3.2 Study Population and Maintenance

Our colony of bed bugs (*Cimex lectularius*) was derived from four natural infestations collected in Southern Ontario between October 2019 and January 2020. We housed our colony in two large 54 x 40 x 40 cm plastic storage bins kept at 27 ± 0.5°C at 60% relative humidity with lights off at 0800 hours and on at 1600 hours. In each plastic bin, we kept bed bugs in 85 mL spice jars each containing several strips of folded filter paper to provide a rough surface for walking and oviposition. Each jar housed approximately 50 to 150 bed bugs of similar life stages, with adults always housed with other adults. Every week, we fed the colony during their dark photoperiod under red light with defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) and using a Hemotek membrane-feeding system (Discovery Workshops, Accrington, UK). In all experiments, we generated virgin bed bugs

by individually isolating recently fed fifth instar bed bugs and grouping them into same-sex groups once they emerged as adults. We marked all focal bed bugs one at a time by briefly anesthetizing them with CO_2, then fastening them onto a wedge-shaped sponge with a single strand of hair. Once secured, we used a toothpick to apply a unique number ID to each bed bug using white paint from a Sharpie oil-based paint marker.

4.3.3 Experiment 1: Effect of social and sexual experience on male sexual competency

Experience phase

All three of our experiments consisted of an eight-day long experience phase followed by a two-day test phase. In Experiment 1, we assessed the general effect of differential social environments on focal male bed bugs. This meant that males from social vs. isolated treatments differed in their exposure to male and female conspecifics and thus also differed in insemination status prior to the observation phase. To generate focal male bed bugs, we marked and fed a group of one day old virgin males, then randomly assigned six males to the social treatment and six males to the isolated treatment. We placed each social focal male in a 15 cm petri dish lined with filter paper along with three age-matched virgin males and four age-matched virgin females (Fig. 4.1a). We placed each isolated focal male in an identical arena as the social males but with no other bed bugs. In each arena, we also placed a wooden shelter constructed from a 25 x 75 x 3 mm balsa wood slat segment covered with a glass microscope slide. Each wood segment contained a 15 x 30 mm cavity with a narrow 5 mm entrance (Fig. 4.1). Our previous studies have shown that bed bugs readily seek refuge, form aggregations, and engage in sexual interactions within such shelters (Yan et al., under review; Yan and Dukas 2022). We also generated age-matched virgin females for focal males to interact with during the test phase by group-housing 24 females in 15 cm petri dish arenas with four balsa wood shelters. Since bed bug reproductive behaviour is closely tied to feeding, we fed all focal males on the last day of the experience phase by briefly (<15 minutes) grouping individuals by treatment, then returning each bed bug to its

respective arena. We also fed all females that were used in the experiment on the last day of the experience phase.

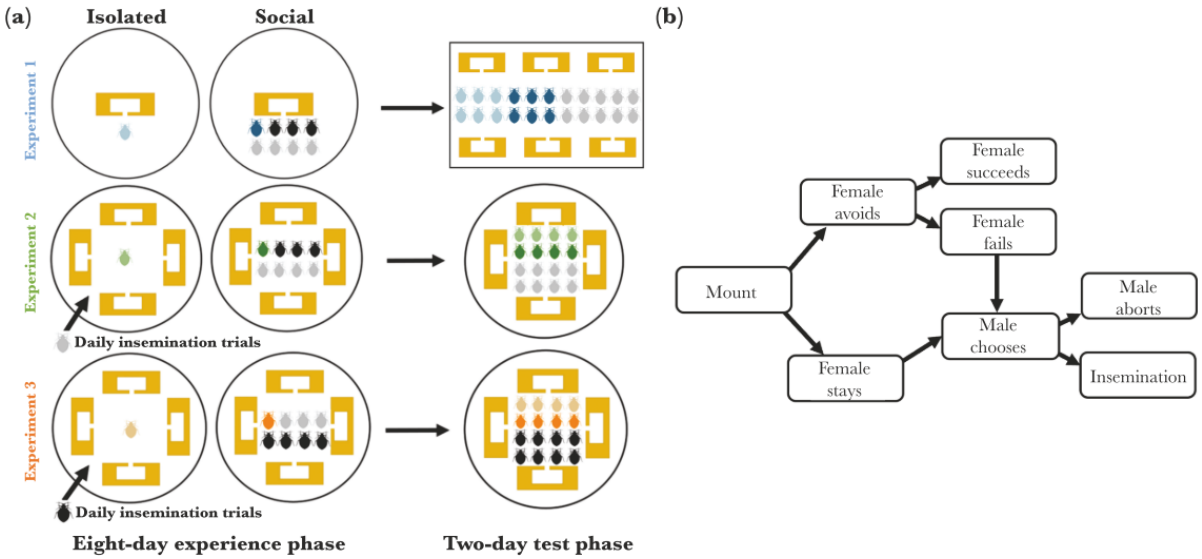

Figure 4.1. (a) Schematic overview of experimental designs. In Experiment 1, we tested isolated (light blue) vs. socialized (dark blue) males in a competitive environment following an 8-d experience phase. In Experiment 2, we tested isolated (light green) vs. socialized (dark green) males but with additional daily insemination trials for isolated males to control for insemination experience. In Experiment 3, we tested isolated (light orange) vs. socialized (dark orange) females and again controlled for insemination experience. For all experiments, gray bed bugs represent standard age-matched virgin females and black bed bugs represent standard age-matched virgin males. (b) Flowchart illustrating how we scored sexual interactions and their outcomes.

Test phase

Following the eight-day experience phase, we placed the six social males and six isolated males along with twelve new individually marked, age-matched virgin females into a 34.5 x 23.5 x 15cm Plexiglass rectangular arena (Fig. 1). Each individual social male was obtained from a different social experience arena. The arena was lined with filter paper and fitted with six brand new balsa wood shelters, identical to those used in the experience phase. We released the bed bugs at the centre of the arena at the start of their dark photoperiod (0800 hours) and an observer continuously scored all sexual interactions in real time for the entire duration of their dark photoperiod (0800-1600 hours) for two consecutive days. We opted not to document interactions during the light photoperiod as bed bugs are nocturnal and therefore largely inactive during the light phase (Mellanby, 1939; Romero et al., 2010). Furthermore, our previous data indicate that greater than 85% of sexual interactions occur during bed bugs' dark photoperiod (Yan & Dukas, 2022). We recorded all mounts and inseminations along with the identities of the individuals involved in each interaction and the outcomes of each interaction according to the flowchart in Figure 1b. We ensured that observers were always blind to male treatment.

Both mounting and traumatic insemination are highly stereotyped and distinctive behaviours in bed bugs. A mount consists of a male 'jumping' onto another bed bug and then dismounting typically within 5 seconds (Stutt & Siva-Jothy, 2001). Male bed bugs are known to frequently mount but rarely inseminate other males (Rivnay, 1933; Ryne, 2009). An insemination is characterized by a male mounting a female, then remaining securely attached with his abdomen curled underneath the female's right abdomen for up to 5 min (1-5 min, Carayon, 1966, p. 103; 30-300 s, Figure 2 in Siva-Jothy & Stutt, 2003). In a data set including 193 insemination durations recorded in our laboratory for another experiment, the average ± 1 SD insemination duration was 102.4 ± 53.9 seconds and the range was 18-406 seconds, with only one insemination lasting less than 20 seconds (Yan & Dukas, 2022). Therefore, we chose 20 seconds as the minimum duration for a mount to be considered an insemination. If males appeared to voluntarily dismount a female before getting into insemination position or within the first 20 seconds of being in insemination position, we

considered the mount to be aborted by the male. We scored female avoidance attempts based on whether females attempted to run away or displayed the refusal posture in response to a mount (Siva-Jothy, 2006), and avoidance success based on whether the female successfully prevented the mounting male from assuming insemination position.

In addition to live, continuous scoring of sexual interactions, we also examined bed bugs' social associations by performing scans where we documented the location of each bed bug. Because the roofs of our shelters were constructed with clear microscope slides, we could readily determine the location of each bed bug without causing disruption. We later used this information to construct networks based on shared shelter use. We conducted a scan at the start of each hour of the dark phase for a total of eight scans on day one and nine scans on day two. In total, we conducted six replicates of the experiment. We documented clear behavioural abnormalities in two males from the social treatment, one from replicate one and another from replicate two. Both males were unable to inseminate females. We decided to remove them from the analyses before knowing what treatment they belonged to, resulting in a final sample size of 34 for the social treatment and 36 for the isolated treatment.

4.3.4 Experiment 2: Effect of social experience on male sexual competency, controlling for insemination status

Experience phase

Next, we tested the effect of social experience on males while controlling for insemination status. We generated focal male bed bugs by marking and feeding a group of one day old virgin males, then randomly assigned four males to the social treatment and four males to the isolated treatment. We placed each social focal male in a 150 mm petri dish lined with filter paper along with three age-matched virgin males and four age-matched virgin females. We placed each isolated focal male in an identical arena as the social males but with no other bed bugs. Here, the experience phase arenas differed from Experiment 1 in that each arena contained four instead of one wooden shelter. We decided to include more shelters in each arena to better mimic naturalistic environments where individuals may have

to move amongst multiple refuges to locate sexual or social partners and to ensure individual experiences were relevant for the observation phase where we also provided bed bugs with multiple shelters. To accommodate the greater number of shelters, we reduced the dimensions of the wooden slat from 75 mm long to 40 mm long by trimming off excess wood. The size of the actual shelter cavity and entrance remained the same.

To roughly equalize the insemination status of social and isolated focal males prior to the observation phase, we conducted controlled and brief insemination trials for isolated males on seven out of the eight experience phase days. We selected seven out of eight days to mirror previously observed rates of traumatic insemination in bed bugs (Johnson, 1941; Stutt & Siva-Jothy, 2001; Yan & Dukas, 2022). For example, when placed in a semi-naturalistic environment with ample room and protective shelters, the average rate of traumatic insemination in bed bugs was 0.89 inseminations per day (Yan & Dukas, 2022). We conducted insemination trials between 1000 and 1200 hours each day by placing each male from the isolated treatment in small, 30 mm petri dishes lined with filter paper along with a single, age-matched female. We continuously observed each pair of bed bugs until the male dismounted the female following insemination and then placed each male back into his respective arena. Most insemination trials lasted less than five minutes. If males did not inseminate females within ten minutes, we added a second age-matched female to their arena. If males still did not inseminate either of the two females after another ten minutes, we returned them to their experience phase arena without completing an insemination. This happened in 12/168 trials and never more than once for the same male, therefore, we continued to use males who missed an insemination in the experiment. Lastly, to ensure that males from the social treatment received equivalent levels of handling, we also briefly removed social males from their experience phase arenas and placed them into 30 mm petri dishes for approximately the same duration of a typical insemination trial before returning them to their respective dishes. On the final day of the experience phase, we fed all focal males by briefly grouping individuals by treatment, then returned each bed bug to their respective arena. We also generated age-matched virgin females for focal males

to interact with during the test phase by group-housing 24 females in 15 cm petri dish arenas with four balsa wood shelters and fed these females on the last day of the experience phase.

Test phase

Following the eight-day experience phase, we placed the four social males and four isolated males along with eight new individually marked age-matched virgin females into a new 150 mm petri dish arena with four wooden shelters, identical to the arenas bed bugs were kept in during their experience phase. We opted to use this arena instead of the large, rectangular Plexiglass arena used in Experiment 1 because we wanted the physical environment to remain consistent between the experience and test phases. Otherwise, all other aspects of the test phase in Experiment 2 were identical to Experiment 1. In total, we conducted six replicates of the experiment resulting in a final sample size of 24 males per treatment.

4.3.5 Experiment 3: Effect of social experience on female sexual competency, controlling for insemination history

Experience phase

Here, we tested the effect of social experience on females' propensity and ability to evade costly inseminations while controlling for female insemination status. We used an identical protocol to Experiment 2 except the focal individuals were now female instead of male. Briefly, we marked and fed focal female bed bugs and then randomly assigned four females to the social treatment and four females to the isolated treatment. We housed social females with three age-matched virgin females and four age-matched virgin males. To roughly equalize the insemination status of social and isolated focal females prior to the observation phase, we conducted controlled and brief insemination trials for isolated females on seven out of the eight experience phase days. If females were not inseminated within ten minutes, we added a second age-matched male to their arena. If either of the two males still did not inseminate the focal female after another ten minutes, we returned the female to their experience phase arena without completing an insemination. This occurred in 7/168 trials

and never more than once for the same female, thus females that received one fewer insemination were still used in the experiment. On the final day of the experience phase, we fed all the focal females by briefly grouping individuals by treatment, then returned each bed bug to their respective arena. To verify that females of the social treatment encountered males frequently, we videorecorded the interactions of four social females with males over a single eight-hour dark photoperiod in replicate two using a Canon VIXIA HF R800 camera. We only examined the dark photoperiod because bed bugs are mostly inactive during their light phase (Mellanby, 1939; Romero et al., 2010). On average, these social females encountered males 12.5 times, were mounted by males 6.25 times, and were inseminated by males 1.25 times over the course of a single dark photoperiod. Lastly, we generated age-matched virgin males for focal females to interact with during the test phase by group-housing 24 males in 15 cm petri dish arenas with four balsa wood shelters and fed these males on the last day of the experience phase.

Test phase

Following the eight-day experience phase, we placed the four isolated females and four social females with eight new individually marked, age-matched, males into a new 150 mm petri dish arena lined with four wooden shelters, identical to the arenas bed bugs were kept in during their experience phase. We ensured that all the males had mated one day prior to the observation phase so that they had some prior sexual experience. The test phase for this experiment was conducted identically to that of Experiment 2 except that we did not record male-male mounts as these interactions did not directly involve the focal females. In total, we conducted six replicates of the experiment resulting in a final sample size of 24 females per treatment.

4.3.6 Statistics

We completed all our analyses in R version 4.1.1 (R Core Team, 2021) and used the package 'glmmTMB' v.1.1.2.2 (Brooks et al., 2017) to construct all our GLMMs (Generalized Linear Mixed Models). We verified all model fits by visually inspecting plots of model

residuals using the 'DHARMa' package (Hartig, 2019) and assessed significance of fixed effects using the *Anova* function from the 'car' package (Fox & Weisberg, 2019). All our GLMMs included treatment, day, and their interaction as fixed factors and replicate and individual ID as random factors. Our analyses were identical for Experiments 1 and 2 as both assessed the effect of social experience on males. For each of these two experiments, we first constructed a GLMM with a binomial distribution to examine whether social experience affected how often males mounted other males as opposed to females. To construct the response variable, we used the *cbind()* function to combine the number of mounts each male directed at males and the number of mounts each male directed at females. Next, we fit a binomial GLMM for each male experiment to examine whether females escaped from previously isolated males at higher rates. Specifically, we constructed the response variable by using *cbind()* to combine mounts that females attempted to but failed to avoid and mounts that females successfully avoided for each male. Then, we constructed a GLMM with number of inseminations secured per male as the response variable for each male experiment. These models were fitted with a Poisson distribution. Since isolated males secured more inseminations than socialized males in both Experiments 1 and 2 despite showing no differences in our various sexual competency metrics, we additionally tested for differences in total mounts performed to see if males from the two treatments differed in sexual motivation. To do this, we constructed GLMMs with total mounts performed by each male (regardless of which sex they were directed at) as the response variable. For Experiment 1, we used a negative binomial distribution since a Poisson model resulted in significant deviations from normality and for Experiment 2, we used a Poisson distribution.

For Experiment 3, which assessed the effects of social experience on females, we first tested whether social experience affected the rate at which females attempted to avoid mounts. To do this, we ran a GLMM using a binomial distribution and the *cbind()* function to combine number of attempted avoidances and number of mounts where females did not attempt to avoid as the response variable. We next assessed avoidance success rate by fitting a GLMM using a binomial distribution and the *cbind()* function to combine number of

successful mount avoidances and number of failed mount avoidances as the response variable. Then, we tested number of inseminations received using a GLMM with a Poisson distribution. Because isolated females were inseminated more frequently than social females on Day 1, despite showing no differences in their propensity or ability to escape mounts, we additionally tested if females were initially mounted by males at different rates. We tested this using a GLMM with number of mounts received as the response variable and a negative binomial distribution since a Poisson model violated assumptions of homogenous variance. Lastly, we also additionally examined rates of males terminating mounts directed at isolated vs. social females. To do this, we used a GLMM with a binomial distribution using *cbind* to combine mounts directed at each female that males terminated, and mounts directed at each female where males had the opportunity to terminate but did not. For all of our statistical models, we used the package 'emmeans' to further test whether the effect of social experience differed between Day 1 and 2 of the test phases if treatment by day interactions were statistically significant.

4.3.7 Social Network Analyses

We constructed social networks in R using the 'igraph' package (Csardi & Nepusz, 2006), where edges represented association indices between dyads based on how often two individuals occupied the same shelter during hourly scans. Specifically, we used the simple ratio index (SRI) to calculate association indices, which is recommended when all individuals can be reliably identified during sampling periods (Hoppitt & Farine, 2018). We then eliminated same-sex connections from association matrices to generate networks that selectively captured opposite-sex associations. Next, we extracted strength values from these opposite-sex networks for each individual. Strength is equivalent to the sum of all edge weights connected to a node and in our networks, represents how often and with how many opposite-sex individuals a bed bug was associated with through shared shelter use.

To test whether social versus isolated individuals differed in opposite-sex network strength, we used a LMM combined with a permutation test for each of the three experiments. Each LMM included strength as the response variable, treatment as a fixed

factor, and replicate as a random factor. Because network measures are inherently non-independent (Croft et al., 2011; Farine & Whitehead, 2015), we performed a node-label permutation test for each experiment by shuffling and redistributing individuals of the focal sex amongst their node positions. For example, in Experiment 1, which assessed the effect of experience on males, we shuffled the network positions of the twelve males in each replicate, while in Experiment 3, we shuffled females amongst their network positions. This type of randomization, where individuals of the focal sex are shuffled to randomly redistribute them between the two treatment groups, results in social networks representing the null hypothesis that treatment has no bearing on social network position. By performing 1000 iterations of this network randomization process per experiment, we were able to compare model coefficients from observed networks to a distribution of model coefficients representing the null hypothesis that social experience has no bearing on the strength of one's opposite-sex associations. Such permutation tests are the most widely used approach to control for statistical non-independence between individuals from the same social network (Croft et al., 2008, 2011; Farine & Whitehead, 2015).

4.4 RESULTS

4.4.1 Experiment 1: Effect of social and sexual experience on male sexual competency

We did not find an effect of prior social and sexual experience on males' propensity to direct mounts at other males as opposed to females (GLMM: Wald $\chi^2_1 = 0.47$, $p = 0.49$; Fig. 4.2a). Similarly, experience with conspecifics did not generate a significant difference in males' abilities to prevent females from successfully evading mounts (GLMM: Wald $\chi^2_1 = 0.26$, $p = 0.61$; Fig. 4.2b). However, we did detect a marginally significant treatment by day interaction for the number of inseminations secured (GLMM: Wald $\chi^2_1 = 3.72$, $p = 0.05$; Fig. 4.1c), as isolated males secured significantly more inseminations than social males on Day 1 ($t = 3.26$, $p < 0.01$; Mean ± SE: isolated = 2.97 ± 0.25, social = 1.74 ± 0.19) but not Day 2 ($t = -0.249$, $p = 0.80$; Mean ± SE: isolated = 0.81 ± 0.19, social = 0.85 ± 0.13). Likewise, we detected a significant treatment by day interaction for mounts performed

(GLMM: Wald χ^2_1 = 11.56, p < 0.001), again because isolated males performed more mounts than socialized males on Day 1 (t = 4.18, p < 0.001; Mean ± SE: isolated = 19.31 ± 2.14, social = 10.32 ± 1.24) but not Day 2 (z = -0.83, p = 0.41; Mean ± SE: isolated = 5.75 ± 1.15, social = 6.62 ± 0.75). We also found significant day effects, with males from both treatments performing more inseminations (GLMM: Wald χ^2_1 = 38.89, p < 0.001) and mounts (GLMM: Wald χ^2_1 = 69.45, p < 0.001) on Day 1 than Day 2. Lastly, we examined opposite-sex strength scores derived from social networks quantifying shared shelter use patterns and found that previously isolated versus socialized males did not differ in their strength of female associations (p_{rand} = 0.62; Figs 4.2d, 4.2e, S4.1, S4.4a). Detailed results for all the statistical models we ran can be found in the Supplementary materials.

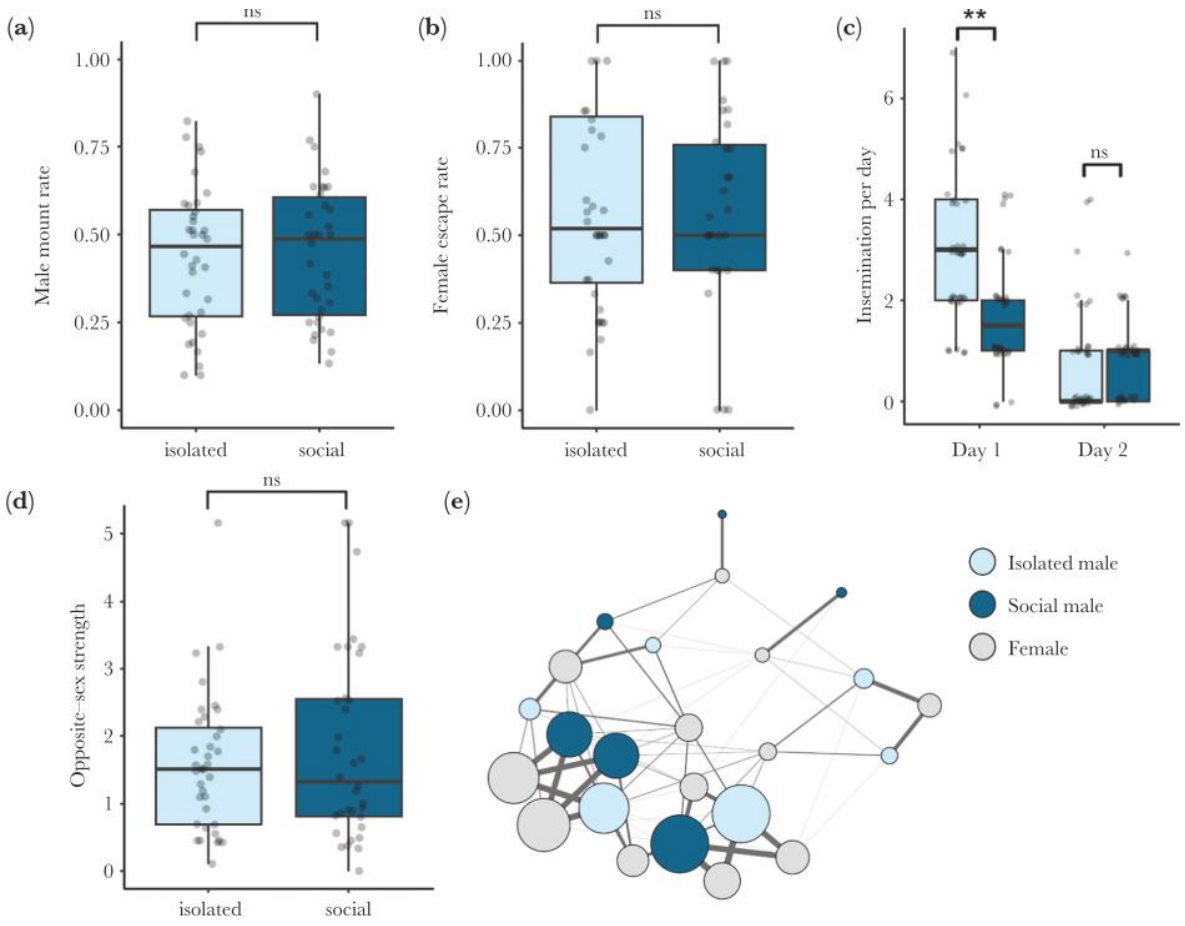

Figure 4.2. (a) Proportion of mounts males directed towards other males, (b) rate of females successfully evading insemination attempts, (c) insemination rates, and (d) opposite-sex association strength for isolated (N = 36) vs. social (N = 34) males. Bold horizontal lines indicate the medians, the boxes represent the interquartile range (IQR) between the first and third quartiles, and the whiskers above and below each box represent values within ±1.5 of the IQR. (e) Weighted opposite-sex social association network from one replicate based on patterns of shared shelter use. Node color corresponds to individual treatment. Edge width represents the strength of association between opposite-sex dyads, and node size corresponds to opposite-sex strength (total sum of edge weights). Social networks for all 6 replicates are depicted in Supplementary Fig. S4.1.

4.4.2 Experiment 2: Effect of social experience on male sexual competency, controlling for insemination status

In Experiment 1, social experience encompassed insemination status since isolated males were completely restricted from access to females and thus entered the test phase as virgins. Experiment 2 differed from Experiment 1 in that we roughly equalized insemination status of males from the two treatments using controlled mating trials. Again, we did not detect differences in social vs. isolated males' propensity to mount other males (GLMM: Wald $\chi^2_1 = 0.31$, $p = 0.58$; Fig. 4.3a) and ability to prevent females from escaping mounts (GLMM: Wald $\chi^2_1 = 0.32$, $p = 0.57$; Fig. 4.3b). However, despite us controlling for insemination status, isolated males secured significantly more inseminations compared to social males across the entire observation phase (Mean ± SE: isolated = 2.52 ± 0.29 inseminations per day, social = 0.87 ± 0.18 inseminations per day; GLMM: Wald $\chi^2_1 = 4.52$, $p < 0.05$; Fig. 4.3c). Therefore, we once again tested whether isolated males performed more mounts overall to examine differences in sexual motivation. Again, we found a significant treatment by day interaction for mounts performed (GLMM: Wald $\chi^2_1 = 7.95$, $p < 0.01$) driven by isolated males performing more mounts than socialized males on Day 1 ($t = 3.32$, $p < 0.01$; Mean ± SE: isolated = 30.67 ± 3.26, social = 22.00 ± 2.39) but not Day 2 ($t = 1.42$, $p = 0.16$; Mean ± SE: isolated = 24.12 ± 4.38, social = 19.38 ± 1.58). Like in Experiment 1, we again detected significant day effects, with more inseminations (GLMM: Wald $\chi^2_1 = 9.44$, $p < 0.01$) and mounts (GLMM: Wald $\chi^2_1 = 44.87$, $p < 0.001$) occurring on Day 1. Lastly, our social network analyses revealed that previously isolated versus socialized males did not differ in how strong their social associations were with females ($p_{rand} = 0.89$; Figs 4.3d, 4.3e, S4.2, S4.4b).

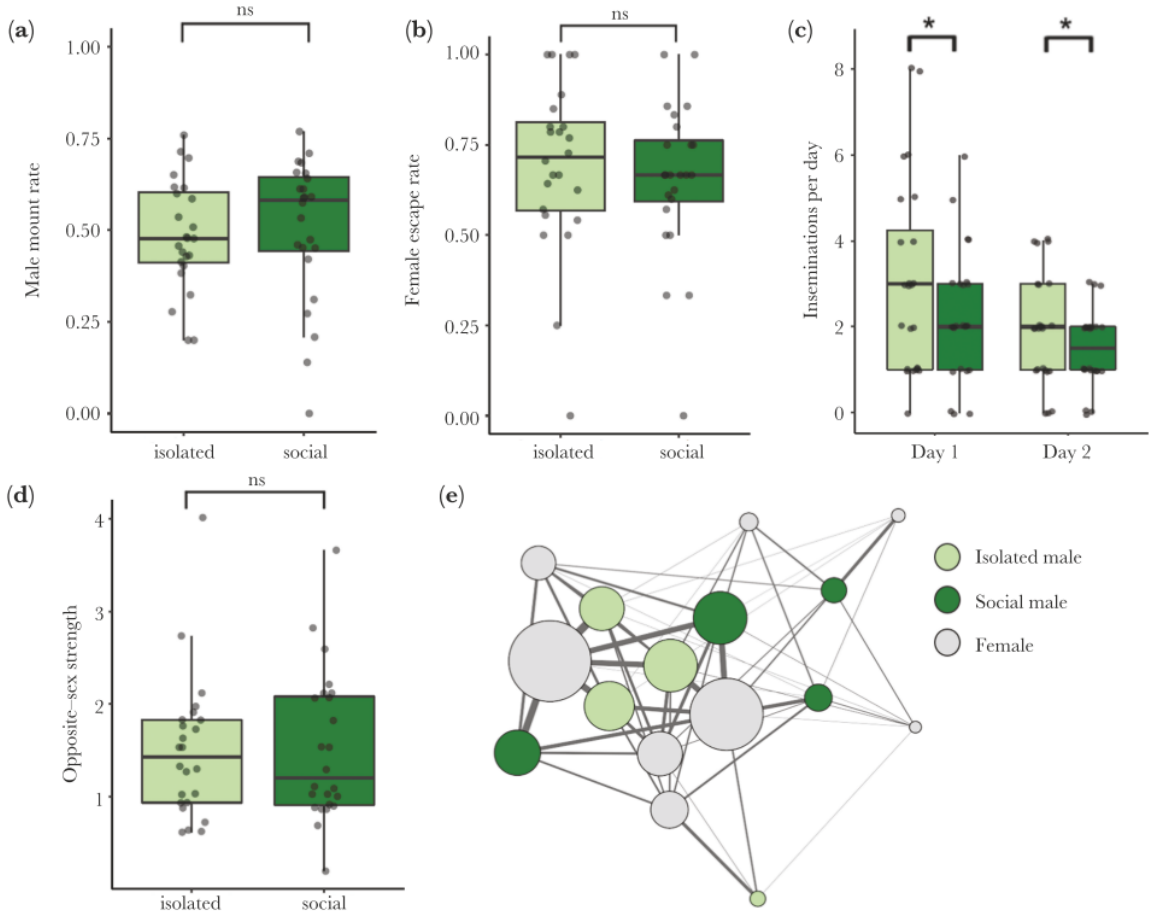

Figure 4.3. (a) Proportion of mounts males directed towards other males, (b) rate of females successfully evading insemination attempts, (c) insemination rate, and (d) opposite-sex association strength for isolated (N = 24) vs. social (N = 24) males. (e) Weighted opposite-sex social association network from one replicate based on patterns of shared shelter use. Node color corresponds to individual treatment. Edge width represents the strength of association between opposite- sex dyads, and node size corresponds to opposite-sex strength (total sum of edge weights). Social networks for all 6 replicates are depicted in Supplementary Fig. S4.2.

4.4.3 Experiment 3: Effect of social experience on female sexual competency, controlling for insemination history

We did not detect an effect of social experience on females' propensity to attempt evading mounts (GLMM: Wald $\chi^2_1 = 0.11$, $p = 0.75$; Fig. 4.4a), females' ability to successfully evade males (GLMM: Wald $\chi^2_1 = 0.33$, $p = 0.56$; Fig. 4.4b), or females' opposite-sex association strength ($p_{rand} = 0.40$; Figs 4.4d, 4.4f, S4.3, S4.4c). However, we did find that females from the isolated treatment were inseminated more than females from the social treatment on Day 1 ($t = 2.37$, $p < 0.05$; Mean ± SE: isolated = 2.21 ± 0.27, social = 1.29 ± 0.29; Fig. 4.4c) but not Day 2 ($t = -0.882$, $p = 0.38$; Mean ± SE: isolated = 0.83 ± 0.20, social = 1.08 ± 0.32; Fig. 4.4c). To explore the mechanism driving this increased insemination rate for isolated females on Day 1, we examined number of mounts received by females and found no evidence of males mounting previously isolated females at higher rates than previously socialized females (GLMM: Wald $\chi^2_1 = 0.36$, $p = 0.55$; Mean ± SE: isolated = 8.40 ± 1.65, social = 7.81 ± 1.37). Instead, we found that on Day 1, socially housed females faced higher rates of rejection by males measured by proportion of mounts that males terminated for each female ($t = -0.84$, $p < 0.01$; Fig.4.4e).

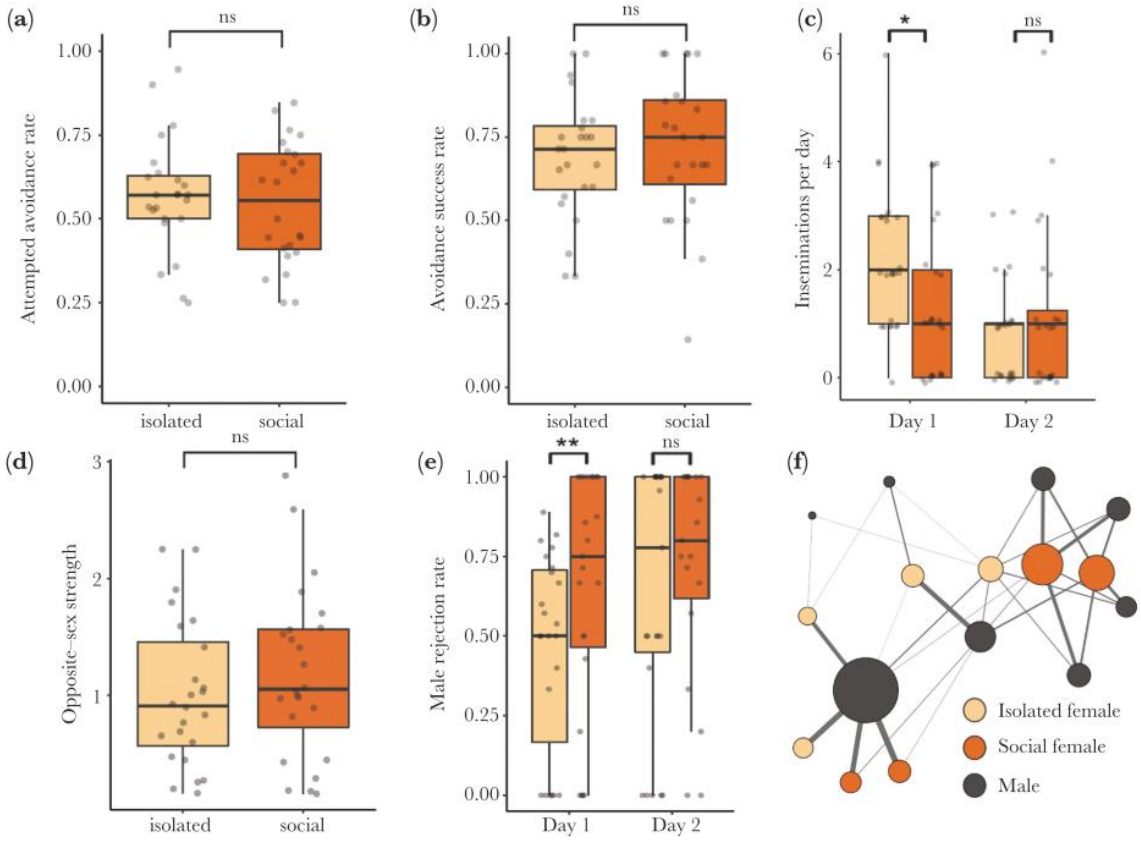

Figure 4.4. (a) Rate of attempting to avoid mounts, (b) avoidance success rate, (c) insemination rate, (d) opposite-sex association strength, and male rejection rate measured for isolated (N = 24) vs. social (N = 24) females. (f) Weighted opposite-sex social association network from a one replicate based on patterns of shared shelter use. Node color corresponds to individual treatment. Edge width represents the strength of association between opposite-sex dyads, and node size corresponds to opposite-sex strength (total sum of edge weights). Social networks for all 6 replicates are depicted in Supplementary Fig. S4.3.

4.5 DISCUSSION

In Experiment 1, we examined how realistic differences in social experience, which encompass differences in mating history, influence various measures of male sexual competency. We found that males provided with social experience did not exhibit improved sexual performance in terms of how often they mounted females as opposed to other males, their abilities to overcome female evasion attempts, and the strength of their opposite-sex social associations (Fig. 4.2). However, on the first day of the test phase, previously isolated males successfully inseminated females at higher rates compared to previously socialized males. We hypothesized that this difference in insemination rate was driven by elevated rates of sexual motivation, which was supported by our finding that males from the isolated treatment displayed higher rates of mounting on Day 1 as well. Therefore, we next conducted Experiment 2 to assess the effects of social experience on male sexual behaviour while controlling for the effect of sexual deprivation. Once again, we found no experience effects on our various sexual performance metrics (Fig. 4.3). Yet, to our surprise, isolated males still mounted and inseminated females at higher rates compared to socially housed males. Thus, our findings from Experiments 1 and 2 suggest that, regardless of their insemination history, male bed bugs that experience social isolation during early adulthood display elevated levels of sexual pursuit and motivation leading to increased insemination success compared to group-housed males. These effects of isolation were short-lived as we only documented differences in inseminations and mounts performed on Day 1, most likely because significantly more sexual interactions occurred on Day 1. Alternatively, the effects of social isolation may have been relatively transient because once introduced to a group, previously isolated individuals rapidly gained social experience.

In natural infestations, which can range from a few to thousands of individuals, bed bugs are typically found in mixed-sex aggregations (Johnson, 1941; Reinhardt & Siva-Jothy, 2007). They are also strongly attracted to the social cues of conspecifics (Levinson & Bar Ilan, 1971; Siljander et al., 2007; Yan & Dukas, 2022). Therefore, though we provided isolated males with an insemination opportunity each day, the lack of conspecifics and social cues of other bed bugs in isolated males' housing arenas may have led to

perceived mate scarcity. Perceived low mate availability is known to alter courtship, aggression, mate-guarding behaviour, and intra-sexual competition in a variety of species (Colwell & Oring, 1988; Emlen & Oring, 1977; Mitani et al., 1996; Wacker et al., 2013; Weir et al., 2011). In species where the social landscape can be highly variable, it would be beneficial for males to adjust their mating strategies when perceived mate availability is low and to increase sexual pursuit intensity and persistence when encountering potential reproductive opportunities. On the other hand, when mate availability is perceived to be abundant, males may adopt a more conservative mating strategy where they expend their energy, sperm, and seminal fluid reserves more prudently, placing greater investment into somatic maintenance or higher quality reproductive opportunities. This explanation is supported by the fact that males of many species, including bed bugs, are known to experience sperm and/or seminal fluid depletion (Birkhead, 1991; Linklater et al., 2007; Preston et al., 2001; Reinhardt et al., 2011; Wedell et al., 2002) and exhibit choosiness based on female mating status (Cook & Gage, 1995; Wedell, 1992; Yan et al., under review).

The higher rates of mounting and insemination seen in previously isolated males could also be driven by decreased sexual motivation and sexual pursuit intensity in social males as a response to female rejection. Similar experience effects are well-documented in other species. For example, courtship conditioning is a phenomenon observed in fruit flies (*Drosophila melanogaster*) where exposure to mated, unreceptive females supresses male courtship behaviour even once the males are introduced to receptive virgin females (Raun et al., 2021; Siegel & Hall, 1979). These experience-based shifts in sexual behaviour are mediated by both behavioural and chemical cues (Dukas et al., 2020; Siwicki et al., 2005). In bed bugs, females are known to display distinct avoidance behaviours and males can also readily differentiate between recently mated and virgin females even based on only residual social cues from previously occupied shelters (Yan & Dukas, 2022). Therefore, the lower mount and insemination rates seen in our socially housed males may suggest that encountering clear rejection signals from females decreases the probability of males investing energy into pursuing potential mates. Further experiments that assess the long-

term fitness consequences of such decreased mount and insemination rates are needed to assess whether responses to rejection are evolutionarily adaptive.

A third, non-mutually exclusive explanation for why isolated males secured a greater number of inseminations is that the presence of rival males during the experience phase caused social males to shift their reproductive investment into post-copulatory traits to account for sperm competition, thus resulting in decreased pre-copulatory success rates. Cues of rivals are known to induce plastic shifts in mating behaviours and reproductive tactics in males of many species (Auld et al., 2015; Bretman et al., 2010a, 2011; Gross, 1996; Noguera, 2019). Furthermore, males that can alter their pursuit behaviour according to local levels of competition are predicted to outperform rival males with fixed pursuit strategies (Fawcett & Johnstone, 2003; Härdling & Kokko, 2005). However, it is worth nothing that currently, very little is known about post-copulatory sexual selection mechanisms in bed bugs. To disentangle the potential effects of perceived low mate availability, experience with female rejection, and presence of rival males, future experiments could expose focal individuals to either only males or only females and assess the relative importance of experience with same versus opposite sex conspecifics.

Unlike in other species (Dukas, 2004; Hoefler et al., 2010; Jordan & Brooks, 2012), experience did not narrow males' range of pursuit towards receptive individuals as males from both treatments generally mounted other males as often as they did females. This lack of improvement supports previous assertions that male bed bugs are incapable of discriminating between male and female conspecifics prior to physical contact (Rivnay, 1933; Siva-Jothy, 2006). Because bed bugs are nocturnal and inhabit tight crevices with patchy distributions of conspecifics, opportunities for pre-copulatory mate assessment may be limited, thus resulting in high rates of same-sex mountings. Red flour beetles (*Tribolium castaneum*) similarly inhabit dark environments and exhibit high rates of male-male sexual behaviour (Levan et al., 2009). However, social experience does reduce males' tendency to display same-sex behaviour in flour beetles (Martin et al., 2015). Importantly, male-male pairings in flour beetles often involves the transfer of a spermatophore and therefore requires considerable energetic investment (Martin et al., 2015). Since bed bugs do not

appear to perform any courtship behaviours and rarely inseminate other males, the time and energy costs associated with directing sexual pursuit towards other males is minimal, especially compared to species with extensive courtship. In such cases where males do not display courtship behaviour, there may be little to no benefit in possessing strong sex discrimination mechanisms prior to mounting.

We similarly did not find experience effects on males' capabilities of overcoming female avoidance. It is therefore possible that, at least in bed bugs, the ability to prevent females from resisting mounts is a fixed trait determined by anatomical or physiological differences that affect mounting speed or grasping strength as opposed to a behaviourally plastic trait. Interestingly, sexual experience similarly does not appear to impact a male's ability to successfully mate in eastern mosquitofish (*Gambusia holbrooki*), another species well-known for its coercive mating system (Iglesias-Carrasco et al., 2019). Currently, it remains unclear why some species show strong effects of experience on sexual competence while others do not. One possibility is that experience may be more beneficial to males in mating systems that involve complex suites of behaviours related to courtship or competition compared to systems characterized by high sexual conflict and persistent harassment.

In females, we similarly did not detect differences between isolated and social individuals' propensity to avoid mounts and success in avoiding mounts. While social females were inseminated more than isolated females on Day 1, our additional analyses revealed that this difference was driven by males terminating mounts directed at social females at higher rates instead of social females displaying increased sexual competency. Most likely, chemical cues indicating high previous sexual encounter rates made the social females less attractive to males. We had expected social females to attempt evading mounts at higher rates due to exposure to frequent harassment from males during their experience phase and the fact that sexual harassment is known to negatively impact the fitness of females in bed bugs and many other species (Helinski & Harrington, 2012; Okada et al., 2017; Réale et al., 1996; Sakurai & Kasuya, 2008; Saveer et al., 2021; Yan et al., under review). Furthermore, while the ability to successfully evade males could be constrained

by physical or physiological limitations and therefore be behaviourally inflexible, we have previously demonstrated that female bed bugs increase male evasion after they experience a few inseminations (Yan et al., under review). Thus, the lack of difference in attempted avoidance rate between previously isolated versus social females remains puzzling. Moreover, our video recordings of social females during the experience phase revealed fairly frequent male encounter (12.5 times per dark period) and mount rates (6.25 times per dark period), meaning that social females received considerably more experience with males compared to females in the isolated treatment. Nonetheless, it remains possible that even higher rates of encountering males and receiving harassment are necessary to generate clear differences in females' propensity or ability to evade males. Future experiments can resolve these questions by manipulating the population density, sex ratio, arena size, or availability of refuges to examine the effects of experience under variable environmental conditions.

Lastly, we found that across all three experiments, experience did not predict an individual's position in opposite-sex association networks. Past research has often reported major social deficits in individual animals that have previously experienced social isolation (Harlow et al., 1965; Hesse & Thünken, 2014; Lihoreau et al., 2009). However, a majority of these studies manipulated the rearing conditions of focal animals throughout their developmental period while the bed bugs in our experiments were all reared socially in the pre-adult stages and then assigned to isolated or social conditions during early adulthood. The consequences of social deprivation may vary greatly depending on the timing and duration of isolation throughout an animal's lifetime. As such, it remains entirely possible that juvenile social experience plays a role in adult social and sexual behaviour in bed bugs. Future studies should seek to determine the most critical periods for social enrichment to further our understanding of experience-based effects.

Because the edges of our social networks represented patterns of shared shelter use between males and females, we hypothesized that strong connections to opposite-sex conspecifics would reflect males' ability to locate and retain continued access to insemination opportunities and females' inability to find refuge from males. Social network

centrality has been found to be associated with various fitness measures across several taxa (Beck et al., 2021; Sabol et al., 2020; Turner et al., 2021). Moreover, the degree and strength of opposite-sex associations have been strongly tied to reproductive fitness in males (Dunning et al., 2023) and number of copulations in females (McDonald et al., 2019a). We had predicted that bed bugs would exhibit a similar link between social network position and various measures of reproductive success since access to mates is one of the major proposed benefits of group formation in animals. However, to our surprise, higher opposite-sex network strength did not predict number of inseminations in bed bugs (Yan and Dukas, unpublished data). As a result, despite bed bugs exhibiting sociality via aggregation formation and chemical communication using various pheromones, our current understanding of how or whether network position in social association networks affect reproductive success remains poorly understood. Overall, to advance our understanding of animal sociality, we have to further examine the relationship between social connectedness and fitness in moderately social species.

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SUPPLEMENTARY INFORMATION

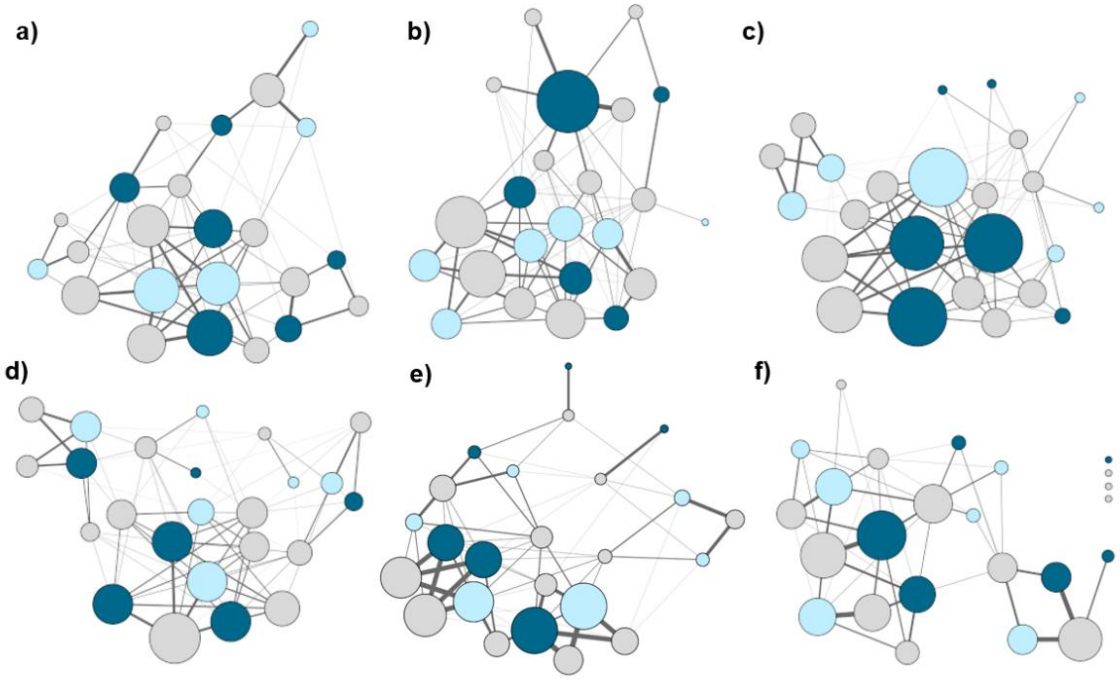

Figure S4.1. Experiment 1 networks. Weighted opposite-sex social association network from all six replicates of Experiment 1 based on patterns of shared shelter use. Node colour corresponds to individual treatment (light blue = isolated male, dark blue = social male, grey = female). Edge width represents the strength of association between opposite-sex dyads and node size corresponds to opposite-sex strength (total sum of edge weights). Nodes with no connections represent individuals that were never observed sharing a shelter with a member of the opposite sex.

Table S4.1. Mixed-effect model results for Experiment 1.

Experiment 1: Effect of social experience on male sexual competency						
Response variable	**Parameter**	**Estimate**	**SE**	**Wald χ^2**	***d.f.***	**p-value**
Rate at mounting other males vs. other females	Treatment	0.1453	0.2122	0.4688	1	0.4936
	Day	0.5411	0.1730	9.7841	1	**0.0018**
	Treatment:Day	-0.5421	0.2609	4.3163	1	**0.0378**
Rate at which female successfully escaped mounts	Treatment	0.0765	0.3337	0.2586	1	0.6111
	Day	0.5356	0.3770	0.5626	1	0.4532
	Treatment:Day	-0.6904	0.5446	1.6069	1	0.2049
Number of inseminations performed	Treatment	-0.5298	0.1623	10.662	1	**0.0011**
	Day	-1.3055	0.2094	38.888	1	**< 0.001**
	Treatment:Day	0.5953	0.3086	3.7200	1	**0.0538**
Number of mounts performed	Treatment	-0.5802	0.1388	17.481	1	**< 0.001**
	Day	-1.2008	0.1441	69.452	1	**< 0.001**
	Treatment:Day	0.7101	0.2086	11.586	1	**< 0.001**

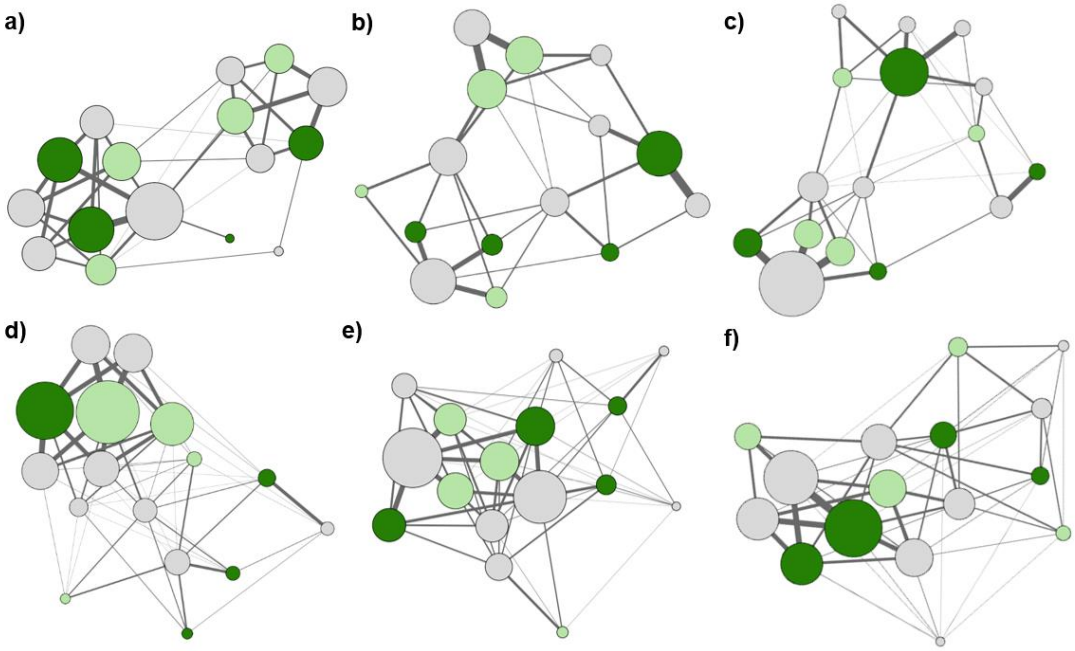

Figure S4.2. Experiment 2 networks. Weighted opposite-sex social association network from all six replicates of Experiment 2 based on patterns of shared shelter use. Node colour corresponds to individual treatment (light green = isolated male, dark green = social male, grey = female). Edge width represents the strength of association between opposite-sex dyads and node size corresponds to opposite-sex strength (total sum of edge weights).

Table S4.2. Mixed-effect model results for Experiment 2.

Experiment 2: Effect of social experience on male sexual competency, controlling for insemination status						
Response variable	**Parameter**	**Estimate**	**SE**	**Wald χ^2**	***d.f.***	**p-value**
Rate at mounting other males vs. other females	Treatment	0.0623	0.2190	0.3112	1	0.5770
	Day	0.0312	0.1182	0.7897	1	0.3742
	Treatment:Day	0.1165	0.1824	0.4079	1	0.5230
Rate at which female successfully escaped mounts	Treatment	-0.0766	0.3540	0.3213	1	0.5708
	Day	0.1294	0.2909	1.5911	1	0.2072
	Treatment:Day	0.3875	0.4622	0.7031	1	0.4017
Number of inseminations performed	Treatment	-0.3151	0.1790	4.5195	1	**0.0335**
	Day	-0.4539	0.1865	9.4445	1	**0.0021**
	Treatment:Day	0.04845	0.2848	0.0290	1	0.8649
Number of mounts performed	Treatment	-0.4182	0.1259	11.015	1	**< 0.001**
	Day	-0.3625	0.0539	44.871	1	**< 0.001**
	Treatment:Day	0.2355	0.0832	7.9510	1	**0.0048**

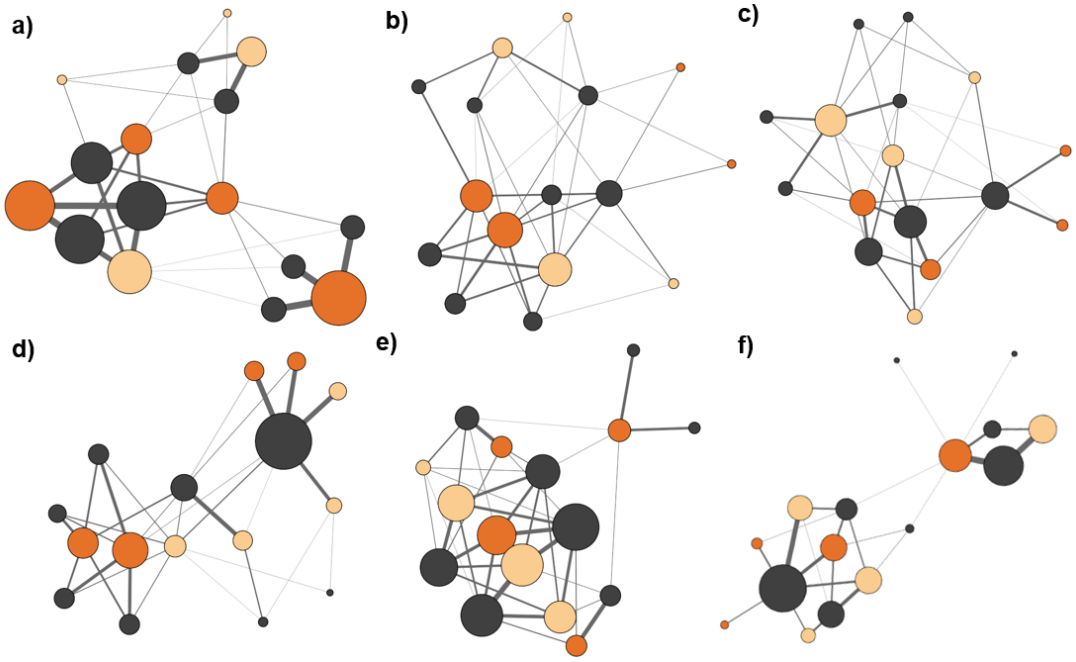

Figure S4.3. Experiment 3 networks. Weighted opposite-sex social association network from all six replicates of Experiment 3 based on patterns of shared shelter use. Node colour corresponds to individual treatment (light orange = isolated female, dark orange = social female, black = male). Edge width represents the strength of association between opposite-sex dyads and node size corresponds to opposite-sex strength (total sum of edge weights).

Table S4.3. Mixed-effect model results for Experiment 3.

Experiment 3: Effect of social experience on female sexual competency, controlling for insemination status						
Response variable	**Parameter**	**Estimate**	**SE**	**Wald χ^2**	***d.f.***	**p-value**
Proportion of mounts females attempted to avoid	Treatment	-0.3010	0.2268	0.1054	1	0.7454
	Day	-0.1356	0.2142	0.4925	1	0.4828
	Treatment:Day	0.5434	0.3107	3.0598	1	0.0802
Proportion of avoid attempts that were successful	Treatment	0.42152	0.40521	0.3331	1	0.5639
	Day	-0.01957	0.33191	0.8649	1	0.3524
	Treatment:Day	-0.47651	0.50013	0.9078	1	0.3407
Number of inseminations received	Treatment	-0.5363	0.2261	5.6258	1	**0.0176**
	Day	-0.9746	0.2624	13.791	1	**< 0.001**
	Treatment:Day	0.8364	0.3717	4.5697	1	**0.0325**
Number of mounts received	Treatment	-0.1044	0.2124	0.3609	1	0.5480
	Day	-0.2520	0.2021	2.7454	1	0.0975
	Treatment:Day	0.0116	0.3004	0.0015	1	0.9691
Rate of rejection by males	Treatment	0.8444	0.3020	2.7066	1	0.0999
	Day	1.1844	0.3246	7.3355	1	**0.0068**
	Treatment:Day	-1.1296	0.4619	5.9818	1	**0.0144**

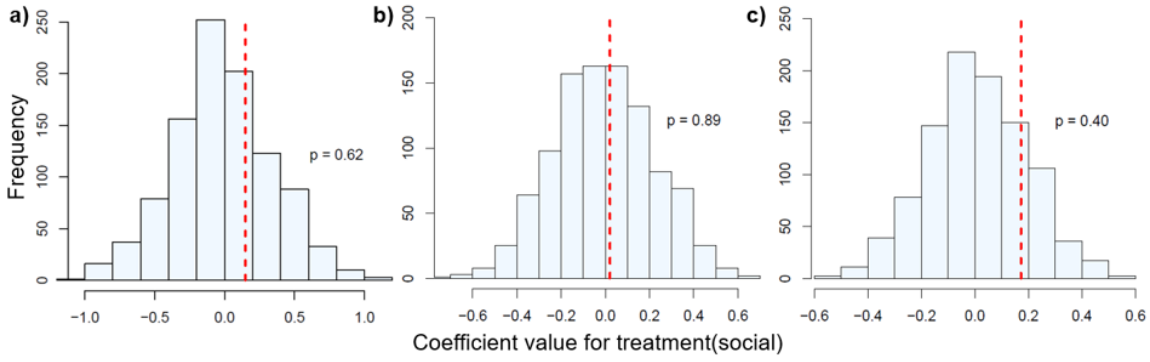

Figure S4.4. Null distributions of LMM model coefficients representing the effect of treatment on opposite-sex strength for (a) Experiment 1, (b) Experiment 2, and (c) Experiment 3. Null distributions for each treatment are the result of 1000 node-label network randomizations. Red dashed lines are model coefficients representing the observed effect of treatment on opposite-sex strength for each experiment.

CHAPTER 5 – OPTIMAL POLYANDRY IN FRUIT FLIES

Yan, J.L., Rosenbaum, J.R.*, Yang, D.*, Dukas, R. (under review). Optimal polyandry in fruit flies.

5.1 ABSTRACT

The study of polyandry has received increasing scientific attention with an emphasis on the fitness benefits and costs that females derive from mating with multiple males. Our understanding of how polyandry affects female fitness, however, remains limited as most previous studies compared the fitness outcomes of a single mating vs. two or three matings and did not separate the consequences of multiple mating from the well-established costs of sexual harassment. To address these gaps, we conducted controlled mating trials with female fruit flies (*Drosophila melanogaster*) that could mate at either low (every eight days), medium (every four days), or high (every other day) rates while controlling for exposure to harassment from males. We found that female lifetime fitness was highest under the high condition followed by the medium mating-rate conditions. Moreover, we did not detect reductions in lifespan as a consequence of higher rates of polyandry. Our results demonstrate that even at realistically high rates, polyandry can lead to net fitness benefits for females, which can have major implications for sexual selection. Specifically, we discuss the significance of our findings as they relate to competition and the evolution of secondary sex characteristics in females and sperm competition amongst males.

5.2 INTRODUCTION

In formulating the concept of sexual selection as a force distinct from natural selection, Darwin (1871) focused on two general observations. The first was that males in many taxa possess what he termed secondary sexual characteristics, which either serve in intra-male competition for females, or make males more attractive to choosy females. The second observation was that it is more often the males that pursued females, which frequently are reluctant to mate. Only several decades later, Bateman (1948) provided the ultimate

explanation for Darwin's observations. By definition, males produce small gametes and females make large gametes. Hence because males are able to produce numerous gametes, they can generally enhance their fitness by mating with an increasing number of females (Fig. 1). Females, on the other hand, are limited by the number of viable offspring they can produce, and thus require only one or a few mates.

Studies using a variety of protocols in a wide range of species have confirmed the early insights of Darwin and Bateman. Sexual selection is indeed typically stronger in males than in females (Janicke et al., 2016). Nevertheless, sexual selection also operates on females, and in some species, females also possess secondary sexual characteristics (Andersson, 1994; Clutton-Brock, 2009; Fromonteil et al., 2023; Hare & Simmons, 2019). To better understand the forces of sexual selection that operate on females, however, we need better information about females' optimal mating rates. A combination of first principles, behavioral observations and experimental data suggest that, in most species, female fitness would show either a decelerating increase or an inverted U shape as a function of mating rate (Fig. 5.1).

With rare exceptions, the sperm to egg ratio is very high, meaning that females can ensure egg fertilization under very low mating rates (Birkhead et al., 2008; Ridley, 1988). A few other factors, however, influence the optimal mating rate, and their combined outcomes would thus determine the shape of female fitness as a function of mating rates. First, mating in some species includes male-provided nuptial gifts of nutritional or defensive value, which females either eat or absorb through their genital tract. Nuptial gifts can increase female fecundity and longevity (Arnqvist & Nilsson, 2000; Gwynne, 2008; Vahed, 1998). Second, some substances in the seminal fluid stimulate females to increase feeding and egg laying while other substances harm females (Avila et al., 2011; Chapman et al., 1995; Chapman, 2001; Chen, 1984; Civetta & Clark, 2000; Gillott, 2003; Hopkins & Perry, 2022; Rice, 1996; Worthington & Kelly, 2016). Third, intromission may lead to physical injury (Blanckenhorn et al., 2002; Crudgington & Siva-Jothy, 2000a; Dukas & Jongsma, 2012; Tong et al., 2021), and pathogen transmission (Hurst et al., 1995; Knell & Webberley, 2004; Lockhart et al., 1996). Fourth, mating could lead to additional costs to

females including increased predation and lost feeding opportunities (Daly, 1978; Magnhagen, 1991; Rowe, 1994; Wing, 1988). Finally, females may gain indirect benefits from mating with multiple males, which would lead to higher offspring survival or fertility (Jennions & Petrie, 2000; Simmons, 2005; Slatyer et al., 2012; Snook, 2014).

Overall then, depending on the balance between benefits and costs of matings, we expect female fitness as a function of mating rate to either approach an asymptote due to diminishing returns of the benefits of additional matings, or reach a peak and then decline if the benefit function decelerates at a faster rate than the cost function (Fig. 5.1). For three major reasons, however, we still lack critical data for testing the hypothetical curves depicted in Figure 5.1. First, an overwhelming majority of experimental studies on polyandry had only two treatments – singly mated females vs. females mated more times, often twice or thrice (Arnqvist & Nilsson, 2000; South & Lewis, 2011). Such studies critically established that a single mating is often sub-optimal for female fitness. However, without a minimum of three treatments, where females are mated to males at higher rates that better reflect polyandry in nature (Bretman & Tregenza, 2005; Haddrill et al., 2008; Imhof et al., 1998; Turnell & Shaw, 2015), our understanding of optimal female mating rates remains limited. Moreover, the handful of studies that varied female mating rates with at least three treatments have generated mixed findings (Arnqvist et al., 2005; Lange et al., 2012; Priest et al., 2008).

Second, and perhaps most importantly, the vast majority of polyandry studies have not separated the fitness consequences of multiple matings from the known costs to females from male harassment. Sexual harassment involves unrelenting male pursuit of females, mountings, and occasionally coercive matings, which can significantly reduce female fitness (Dukas & Jongsma, 2012; Partridge & Fowler, 1990; Rice et al., 2006; Sakurai & Kasuya, 2008; Saveer et al., 2021). That is, the easiest way to manipulate mating rates is by varying either the duration over which females are exposed to males or varying the number of males that females are continuously exposed to. This, however, means that the cost of multiple matings is confounded by the cost of longer duration of male harassment. Even when exposure to harassment is controlled through the use of males with their

genitalia ablated or glued (Fowler & Partridge, 1989; Jigisha et al., 2020; Siva-Jothy & Stutt, 2003), the high costs induced by continuously housing individual females with several males may override any potential fitness benefits of polyandry. Therefore, as it currently stands, the fitness consequences of polyandry, in the absence of excessive sexual harassment, remain unclear.

Third, a large proportion of existing studies on how multiple mating influences female fitness do not distinguish between true polyandry, where females mate with multiple males, and repeated mating with the same male (Arnqvist & Nilsson, 2000; Slatyer et al., 2012; South & Lewis, 2011). While one might expect the effects of polyandry and repeated monandrous mating to be similar, the two forms of multiple mating can largely differ in how they impact female fitness (Slatyer et al., 2012). In ground crickets (*Allonemobius socius)*, for example, mating four times to the same male results in fitness benefits for females while mating once to each of four distinct males leads to fitness reductions in comparison to a single mating (Fedorka & Mousseau, 2002). Unlike when females mate repeatedly with the same male, true polyandry leads to sperm competition, where males may respond to the presence of rivals' sperm by adjusting the amount or composition of ejaculate transferred during mating in a way that alters the females' physiology, reproduction, and behaviour (Firman & Simmons, 2008; Parker, 1970; Slatyer et al., 2012). Hence, in order to quantify optimal polyandry, we need to vary the rate of mating with distinct males.

To critically test the relationship between polyandry and female fitness, we tracked the lifetime reproductive success of female fruit flies (*Drosophila melanogaster*) assigned to either low (0.125 matings/day), medium (0.25 matings/day), or high (0.5 matings/day) rates of matings with distinct males. These rates reflected a realistic range of likely polyandry in the field. Female fruit flies can store about 500 sperm (Manier et al., 2010) and may lay up to several dozen eggs per day (Klepsatel et al., 2013; Markow, 2000; Shapiro, 1932). This means that they might have to mate every several days in order to sustain fertility (Markow et al., 2012). While field estimates of mating rates in fruit flies vary owing to genetic variation (Pyle & Gromko, 1981) and distinct methods of estimation,

they range between 0.2 to 0.9 matings per day (Dukas, 2020; Giardina et al., 2017; Pyle & Gromko, 1978). Throughout our mating trials, we made sure to both minimize and equalize the negative effects of sexual harassment across treatments through short, controlled exposures to virgin males. Based on the multiple factors discussed above, we predicted that mating with multiple males would decrease female lifespan and thus expected females in the low mating rate treatment to live the longest followed by females in the medium mating rate treatment. However, we expected that higher rates of mating with multiple males would lead to fecundity benefits. Therefore, we expected females in the medium mating rate treatment to exhibit the highest lifetime reproductive success, reflecting an optimal intermediate rate of polyandry, where females gain fecundity benefits from having multiple mates while mitigating the lifespan reducing costs of excessive mating.

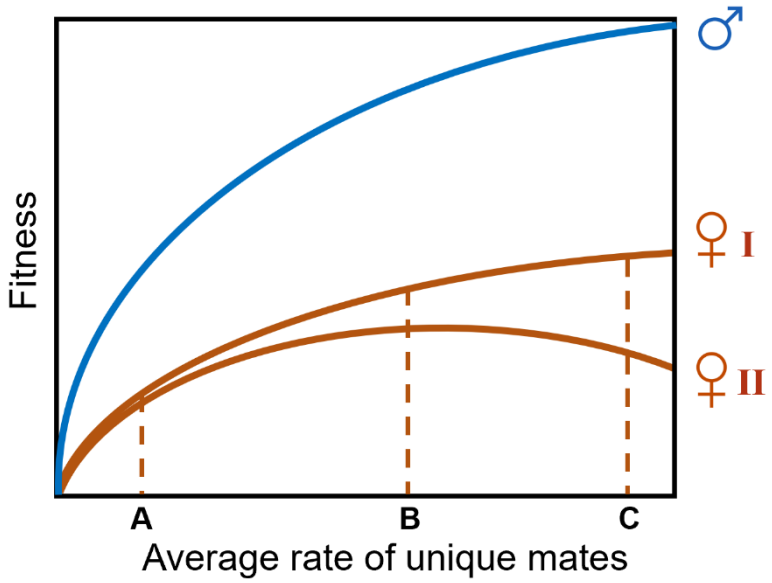

Figure 5.1. Hypothetical curves illustrating the net fitness consequences of multiple mates in males (blue) and females (red). In males, we generally expect fitness to increase as they mate with an increasing number of females. In females, depending on the balance between the benefits and costs of polyandry, we expect fitness to either asymptote (I) or reach a peak and then decline as a function of the rate of mating with unique males (II). Points A, B, and C reflect the rates of polyandry that studies should aim to capture in order to critically test females' optimal mating rates.

5.3 METHODS

5.3.1 Ethical note

Our research complied with all applicable laws and did not require approval from an ethics committee. While we do not require formal ethics approval, we treat our subjects in accordance with strict animal ethics standards under the assumption that they experience emotion in general and pain in particular.

5.3.2 Population and maintenance

We used a lab population of fruit flies established by 600 wild-caught females collected in Hamilton, ON in 2018. We kept all flies in standard 25 x 95 mm food vials containing 5 ml of our standard fly medium (1 litre food contained water, 90 g of sucrose, 75 g of cornmeal, 10 g of agar, 32 g of yeast and 2 g of methyl paraben dissolved in 20 ml of ethanol). We sprinkled live yeast into vials that housed females to stimulate egg-laying. We maintained all flies in an environmental chamber kept at 25˚C and 60% relative humidity with a 12:12 h light:dark cycle with lights turning on at 1100 hours. To obtain virgin females and males for the experiment, we sexed flies within 10 hours of eclosion under light CO_2 and subsequently handled flies using gentle aspiration.

5.3.3 Mating trials and fitness measures

When focal females were four days old, we mated them each once and then randomly assigned them to either low, medium, or high mating rate conditions. We conducted standard mating trials by aspirating two virgin males into each female's vial. We then reduced the volume of the vial to ~5 mL using a foam plug to promote mating. Mating trials began at 9:00 am and lasted for three hours or until a female mated. Throughout the mating trials, observers continuously scanned each vial to record the start and end of each mating. Once a mating ended or after three hours elapsed since a trial began, we immediately removed both males from females' vials to prevent re-mating. After the first day of mating trials, females were given the opportunity to mate either every two days (high treatment), every four days (medium treatment), or every eight days (low treatment), reflecting a

realistic range of likely polyandry in fruit flies under natural settings (Dukas, 2020; Giardina et al., 2017; Markow et al., 2012; Pyle & Gromko, 1978). In total, we ran two replicates of the experiment with 20 females assigned to each treatment per replicate. However, due to three early deaths, where females died within the first five days of experiment, and one escaped female, our final sample sizes were 37, 39, and 40 for the low, medium, and high treatments, respectively.

The males we used to mate with focal females were always virgin and either all four-day-old or all six-day-old within a given mating-trial day. On days when females from some treatments were to be mated and others not, we controlled for exposure to males by placing two virgin males with glued genitalia into females' vials and again, reducing the volume of each vial with a foam plug. We generated glued males by first anesthetizing a two- to three-day-old virgin male with CO_2. We then fastened one male at a time onto a wedge-shaped sponge using a single strand of hair and applied a tiny drop of superglue using a sewing needle onto the tip of each male's abdomen. We ensured that our gluing procedure did not alter the behaviour of males by conducting preliminary behavioural trials, where we tracked the proportion of time glued vs. normal males courted virgin females during a 15-minute trial. We did not detect any differences in courtship rate between glued vs. normal males. We additionally recorded courtship rates of glued vs. normal males throughout the first replicate of our experiment and did not detect any differences (see Supplementary Materials for further details). Given the known fitness costs of sexual harassment across taxa (Dukas & Jongsma, 2012; Partridge & Fowler, 1990; Rice et al., 2006; Saveer et al., 2021), we ensured that the duration of exposure to glued males for females experiencing only harassment was matched to how long females in mating trials were exposed to unglued males.

In total, we ran mating trials for 32 days, from when focal females were all mated for the first time when they were 4 days old until they were 36 days old. To measure longevity, we inspected each female's vial every morning until all females died. To measure total lifetime reproductive success, we counted the total number of adult offspring produced by each female. During the first 12 days of the first replicate, we moved females into fresh

food vials with live yeast for egg-laying every two days. On day 12, as egg-production rate began to decrease, we moved females into fresh vials every four days. On day 32, once egg-production rate became very low, we moved females into fresh vials every week. Because larval density was slightly higher than expected in replicate one, we moved females into new vials more frequently for replicate two to ensure all larvae had an adequate amount of food to pupate. We therefore moved females into fresh food vials every day for the first 12 days, then every other day until day 20, every four days up until day 32, and every week afterwards. Once we removed females from vials, we continued storing each vial in our incubator for two weeks, allowing adult offspring to eclose. We then froze the vials and let an observer blind to female treatment count the number of adult offspring in each vial.

Lastly, to help us understand whether failures to mate resulted from lack of males' interest in females or females' rejections, we tracked whether courtship directed at females varied by treatment and females' ages throughout both replicates. These data also allowed us to verify that glued males displayed regular levels of courtship. Detailed methods and results for our courtship observations can be found in the Supplementary Materials.

5.3.4 Statistics

We completed all our analyses in R version 4.3.3 (R Core Team, 2024) and used the package "glmmTMB" (Brooks et al., 2017) to run our linear mixed-effect models (LMMs). We verified all model fits by visually inspecting plots of model residuals using the "DHARMa" package (Hartig, 2019) and assessed the significance of fixed effects using the Anova function from the "car" package (Fox et al., 2012). Because females did not always mate when given the opportunity to, mating rates for the medium and high treatments were not as high as we intended. Therefore, to assess if the rates of mating were significantly diverged across treatments, we constructed a linear mixed-model (LMM) with daily mating rate as the response variable, treatment as a fixed factor, and replicate as a random factor. Daily mating rates were calculated as the number of times each female mated over the number of days she was alive during the 32-day period of mating trials.

To compare survivorship between females from the low, medium, and high mating rate treatments, we fit a Cox proportional hazards mixed-effects model using the "survival" and "coxme" packages (Therneau, 2015, 2022). This model included days each female survived as the response variable, treatment as a fixed factor, and replicate as a random factor. We next assessed treatment differences in lifetime reproductive success by constructing a LMM with number of adult offspring produced per female as the response variable, treatment as a fixed factor, and replicate as a random factor.

5.4 RESULTS

While we exposed females to new virgin males at low (0.125 matings/day), medium (0.25 matings/day), or high (0.5 matings/day) rates, the females did not mate at every given opportunity. Nonetheless, mating rates were significantly different across the three treatments (LMM: Wald $\chi^2_2 = 75.08$, $p < 0.001$; Fig. 2a). We did not detect an effect of polyandry rate on female survivorship (Cox mixed-effects regression: $\chi^2_2 = 0.92$, $p = 0.63$; Fig. 2b). However, we found that total lifetime offspring production significantly increased with increasing rates of polyandry (LMM: Wald $\chi^2_2 = 16.49$, $p < 0.001$; Fig. 2c). A visual inspection of the daily average offspring production rates indicated that the low treatment females produced fewer offspring per day the longer it had been since their most recent mating opportunity, and increased offspring production again following their next mating opportunity (Fig. S1). Lastly, we did not detect treatment differences in the rate at which females were courted by males in either replicate one (GLMM: Wald $\chi^2_2 = 0.37$, $p = 0.83$; Fig. S2) or replicate two (LMM: Wald $\chi^2_2 = 1.26$, $p = 0.53$; Fig. S3).

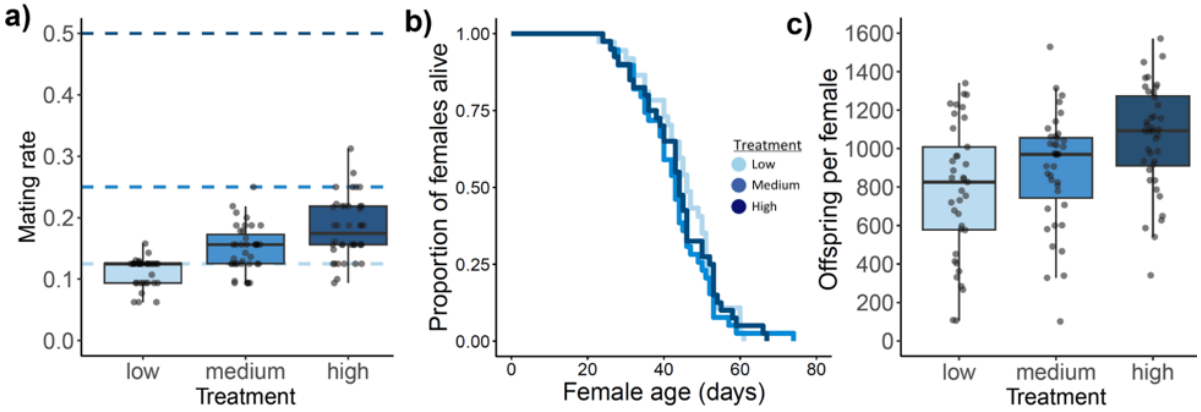

Figure 5.2. a) Observed rates of mating for females assigned to low (N = 37), medium (N = 39), and high (N = 40) mating rate conditions. Bold horizontal lines indicate the medians, the boxes represent the interquartile range (IQR) between the first and third quartiles, and the whiskers above and below each box represent values within ±1.5 of the IQR. Dashed lines represent the intended mating rate for each treatment denoted by line colour. b) Survival curves for females in the low, medium, and high mating rate conditions. c) Total offspring produced by females in the low, medium, and high mating rate conditions.

5.5 DISCUSSION

In our study, we addressed the most common limitations of previous polyandry experiments by assigning females to three increasing rates of mating with unique males, while limiting and controlling for exposure to sexual harassment. We found that higher rates of polyandry led to increased female fitness as the high mating rate females produced the most lifetime offspring followed by the medium mating rate females. Visual examination of daily offspring production rates revealed that low treatment females gradually decreased their offspring production the longer it had been since they last mated, but quickly recovered following their next mating opportunity (Fig. S5.1). This pattern indicated that the low treatment females were depleted of sperm and/or seminal fluid compounds that are known to play a crucial role in reproduction (Chapman, 2001; Hopkins & Perry, 2022; Poiani, 2006). While prior meta-analyses clearly demonstrated that a single mating often does not maximize female fitness (Arnqvist & Nilsson, 2000; Slatyer et al., 2012; South & Lewis, 2011), our results indicate that even low levels of polyandry can be insufficient for females to maximize offspring production. Depletion of essential male ejaculate components that are received by females during mating therefore may act as a strong driver of the evolution of polyandry. Moreover, unlike in our mating trials that exclusively used virgin males, males in natural populations are expected to vary in the status of their sperm and seminal fluid reserves (Birkhead, 1991; Linklater et al., 2007; Preston et al., 2001; Reinhardt et al., 2011; Wedell et al., 2002), further constraining the availability of male ejaculate components for females, thus increasing females' tendencies to mate with multiple males.

Unexpectedly, and contrary to prior studies on multiple mating in fruit flies (Chapman & Partridge, 1996; Priest et al., 2008) as well as prior meta-analyses summarizing the effects of multiple mating on female fitness components across many species of arthropods (Arnqvist & Nilsson, 2000; Slatyer et al., 2012; South & Lewis, 2011), females that mated at higher rates in our experiment did not experience reduced lifespans. Most likely, the discrepancy between our results and past studies documenting high longevity costs of polyandry is owing to the fact that we minimized and equalized the amount of sexual harassment received by females across treatments, while most prior

studies on female multiple mating continuously house females with males. Exposure to sexual harassment, in the absence of mating, has been shown to reduce elements of female fitness across many species (Dukas & Jongsma, 2012; Helinski & Harrington, 2012; Okada et al., 2017b; Partridge & Fowler, 1990; Réale et al., 1996; Rice et al., 2006; Saveer et al., 2021). In fact, Bretman & Fricke (2019) found that mere exposure to males decreased female fruit flies' lifespan, but that receiving sex peptide (SP), a seminal fluid protein often cited as toxic to females (Chen, 1984; Hopkins & Perry, 2022; Wigby & Chapman, 2005), did not induce such longevity costs. Finally, experiments where females are continuously housed with males have been shown to generate significantly lower benefits of polyandry compared to experiments that do not involve excessive sexual harassment through co-habitation with males (Arnqvist & Nilsson, 2000). Therefore, our results, in addition to the well-documented evidence that mere exposure to males can reduce female fitness, suggest that the current estimated costs of polyandry may be inflated, reflecting the costs of sexual harassment rather than the acts of matings themselves.

While the mating rates in our low, medium, and high treatment females were statistically diverged, our medium and high treatment females mated at lower rates than we intended (Fig. 5.2a). Specifically, we provided the medium and high treatments with opportunities to mate every 4 and 2 days, but they mated only about every 6 and 5 days respectively. Female fruit flies can display a wide range of rejection behaviours and, importantly, can ultimately determine whether mating occurs (Connolly & Cook, 2008; Dukas et al., 2020). The fact that females in our medium and high treatments refused many mating opportunities suggests that higher rates of mating could have been undesirable and potentially costly to the females. It is also possible, however, that females' low receptivity is under male control because it is clearly in the males' interest to prevent female remating owing to the strong last male precedence (Gromko et al., 1984; Price et al., 1999). Indeed, it is known that seminal fluid components lead to reduced female receptivity (Chen et al., 1988), although this may serve both female and male interests (Hopkins et al., 2024). In any event, given the limit to mating rates that we could achieve in our experimental system, we cannot determine whether hypothetically higher mating rates would increase or decrease

female fitness. That is, the actual mating rates in our high mating rate treatment may have been at point B in Fig. 5.1, whereas we would require higher mating rates corresponding to point C in Fig. 1 to critically characterize the association between mating rates and female fitness. To better understand the adaptive significance of polyandry and critically test if intermediate rates of polyandry optimize female fitness, future studies should seek out other experimental techniques or animal models that enable the experimenters greater control on polyandry rates. For example, bed bugs (*Cimex lectularius*) are a highly polyandrous species that mates through traumatic insemination (Carayon, 1966; Reinhardt & Siva-Jothy, 2007; Stutt & Siva-Jothy, 2001), thereby perhaps making it more difficult for females to evade matings. In bed bugs, exposing females to males daily results in very high rates of successful traumatic insemination (Yan et al., in press), illustrating that bed bugs or other species with coercive mating systems could help us elucidate the optimal rates of polyandry.

Importantly, decreased interest in recently mated females has been documented in males across the animal kingdom (Stoltz et al., 2007; Thomas, 2011; Wiklund & Forsberg, 1986; Yan et al., in press). Therefore, we observed males' courtship in order to understand if failures to mate in our medium and high treatment females were due to decreased attractiveness to males or females' rejection of pursuing males. Across both replicates, we did not detect differences in the amount of courtship directed at females based on their mating rate treatment (Figs S5.2, S5.3). This suggests that males did not find females in our medium and high mating rate treatments less attractive than the low treatment females.

Overall, our results demonstrate that at least in some species, female fitness is not optimized at low rates of polyandry and instead, females can increase their fitness by mating with multiple males at higher rates. Depending on the ratio of sexually receptive males to females within a given population, these findings can have broad implications for how sexual selection operates on both sexes. The benefits of polyandry for females can lead to competition amongst females for mating opportunities resulting in intensified sexual selection on female secondary sexual characteristics (Clutton-Brock, 2009; Hare & Simmons, 2019; Kvarnemo & Simmons, 2013). We expect this intensified sexual selection on females to be especially strong in mating systems where males invest considerably into

each of their mates, for example, in species with giant sperm (Pitnick et al., 1995; Pitnick & Markow, 1994), nuptial gifts (Gwynne, 2008; Vahed, 1998), or paternal care (Burke et al., 1989; Kempenaers & Dhondt, 1993; Nakamura, 1998). However, in other cases, competition amongst females over access to males may remain low even if high rates of polyandry are beneficial as long as the ratio of sexually available males to females remains sufficiently high (Clutton-Brock & Parker, 1992; Kvarnemo & Simmons, 2013). To better understand the evolutionary drivers of female reproductive traits, future empirical work is needed to examine the relationship between the benefits of polyandry and sexual selection in females.

As noted by Parker (1970), polyandry also results in the temporal overlap of male ejaculates in the female reproductive tract, thus leading to sperm competition amongst males. As a result, when females engage in polyandry, sexual selection not only favours traits that help males secure mating opportunities, but also adaptations that increase males' fertilization success relative to their rivals (Kvarnemo & Simmons, 2013; Parker, 1970; Parker & Pizzari, 2010). For example, males may evolve mate guarding behaviour (Elias et al., 2014; Sakaluk, 1991; Tamura, 1995), mating plugs (Bretman et al., 2010b; Fromhage, 2012; Shine et al., 2000), or seminal fluid proteins that supress female receptivity (Chapman, 2001; Scott, 1986) to prevent females from mating with other males. Polyandry also has the potential to decouple the relationship between males' mating success and fitness, especially in cases with non-random patterns of mating or non-random patterns of sperm usage (Greenway et al., 2021; McDonald & Pizzari, 2017). For example, under positive mating assortment, where more polygynous males tend to mate with more polyandrous females, increased mating success in males is also associated with higher levels of sperm competition (McDonald & Pizzari, 2017). Likewise, in polyandrous mating systems with strong last male sperm precedence, sperm displacement by rival males would be common and as a result, mating with a greater number of females may not necessarily translate to fitness gains for males (Greenway et al., 2021). However, if females typically only accept mates when their stored sperm is nearly depleted (Gromko & Markow, 1993), temporal overlap of male ejaculates may remain uncommon, thus reducing the effects of

polyandry on sperm competition intensity. Therefore, elucidating the optimal patterns of remating for females, in terms of both number of mates and the ideal timing of matings, is essential for understanding the extent to which polyandry mediates pre- and post-copulatory sexual selection in males.

To conclude, our results demonstrate that polyandry can result in fitness benefits for females even when females mate with more than two or three males throughout their lifetime. However, we cannot determine whether higher rates of polyandry than we recorded would result in lower or higher female fitness because mating rates are often under subjects' rather than experimenters' control. Our results also suggest that exposure to high levels of sexual harassment may have inflated previous estimates of the costs associated with polyandry. Overall, the fact that females may gain fitness from higher rates of polyandry suggest that, in many species, sexual selection on females is stronger than previously appreciated.

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5.8 SUPPLEMENTARY MATERIALS

5.8.1 Methods for courtship observations

To track whether courtship directed at females varied by treatment and females' ages, we collected courtship data throughout both replicates. These data could help us understand whether failures to mate results from lack of males' interest in females or females' rejections. These data also allowed us to further verify that glued males displayed regular levels of courtship.

In the first replicate, starting from when females were 14 days old, we continuously scanned all vials during mating and harassment trials. If a male was actively courting a female during a scan, we recorded a tally for the female indicating a courtship bout. Once a female received a minimum of five courtship bouts, we stopped scanning her vial as we determined that males found this female sexually attractive. In the second replicate, we collected detailed courtship observations on a randomly selected subset of females from each treatment. Specifically, we selected three females from each treatment and performed courtship observations for the first 10 minutes of their mating trials on each day where females from all three treatments were to be mated (i.e. when females were 4, 12, 20, and 28 days old). We observed the same nine females, three from each treatment, each time we collected courtship data. To conduct courtship observations, an observer blind to female treatment and ID continuously watched three females at a time, one from each treatment, and live-recorded courtship latency, courtship duration, and mating latency (if mating occurred) using the *Drosophila Assay App.*

5.8.2 Statistics for courtship observations

For the first replicate, once we documented five courtship bouts directed at a female, we considered them to be sexually attractive. Since most females reached five courtship bouts within their mating or harassment trial, we treated courtship received as a binary variable where females either did or not did reach five courtship bouts. We first tested whether glued vs. normal males courted females at different rates throughout our experiment by fitting a generalized linear mixed model (GLMM) with whether females

received five courtship bouts as the response variable, male type (glued vs. normal) as a fixed factor, and female ID as a random factor. Next, we fit a GLMM to investigate whether females became less attractive over the course of the experiment and whether changes in attractiveness differed by female treatment. The response variable for this model was again whether females received five courtship bouts. We included female treatment, age, and their interaction as fixed factors and female ID as a random factor.

For the second replicate, we examined courtship rate based on the proportion of time females were courted by males during their 10-minute observation trials. If females mated during the 10-minute trial, trial duration was shortened to the mating latency. To test if female attractiveness decreased over the course of the experiment and whether changes in attractiveness differed by treatment, we fit a LMM with courtship rate as the response variable, treatment, female age, and their interaction as fixed factors, and female ID as a random factor. If interactions were statistically significant, we used the package *emmeans* to perform post-hoc analyses.

5.8.3 Results for courtship observations

First, we did not find any evidence that glued males courted females any less than normal, unglued males (GLMM: Wald $\chi^2_2 = 0.0003$, $p = 0.99$; Proportion of trials reaching five courtship bouts: glued males = 0.53, unglued males = 0.53). Our courtship data from the first replicate revealed that the proportion of females receiving a minimum of five courtship bouts decreased as they aged (GLMM: Wald $\chi^2_2 = 5.69$, $p = 0.02$; Fig. S2). However, we did not detect differences in courtship received based on female treatment (GLMM: Wald $\chi^2_2 = 0.37$, $p = 0.83$; Fig. S2). We additionally did not detect treatment by age interaction effects, though our data show a slight non-significant trend of high treatment females showing the strongest decline in attractiveness followed by the medium treatment females (GLMM: Wald $\chi^2_2 = 3.28$, $p = 0.19$; Fig. S2).

For replicate two, we performed courtship trials when females were 4, 12, 20, and 28 days old. However, we did not observe any courtship or mating during the 10-minute observation period when females were 12 days old and thus, only included data from females at 4, 20, and 28 days old. We did not detect treatment differences in the rate at

which females were courted by males (LMM: Wald $\chi^2_2 = 1.26$, $p = 0.53$; Fig. S3). Unlike in replicate one, we additionally did not detect an overall effect of females receiving less courtship as they aged (LMM: Wald $\chi^2_2 = 3.49$, $p = 0.17$; Fig. S3). However, there was a significant treatment by female age interaction where females from the low treatment received decreased rates of courtship as they aged (LMM: Wald $\chi^2_2 = 10.20$, $p < 0.01$; Fig. S3).

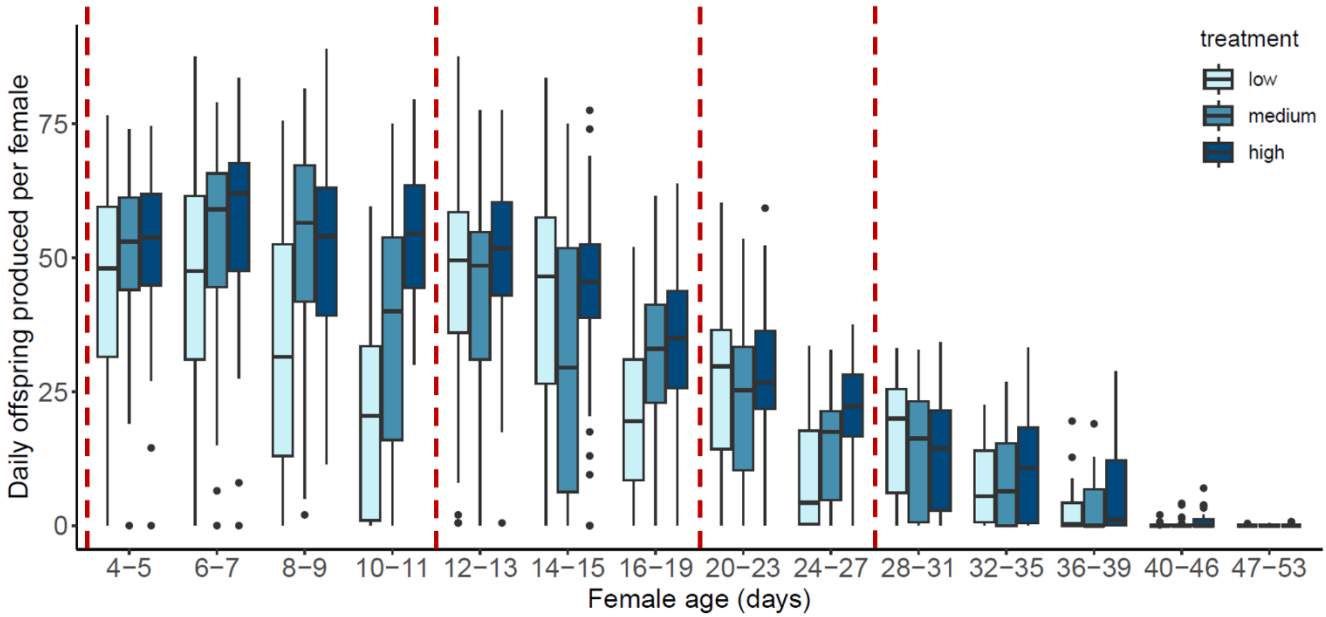

Figure S5.1. Average daily offspring production rates for living females in the low, medium, and high mating rate groups. The initial sample sizes for each treatment are N = 37 (low), N = 39 (medium), and N = 40 (high), but they gradually decrease with female death. Red dashed lines at female ages 4, 12, 20, and 28 correspond to when females from all three treatments were provided the opportunity to mate.

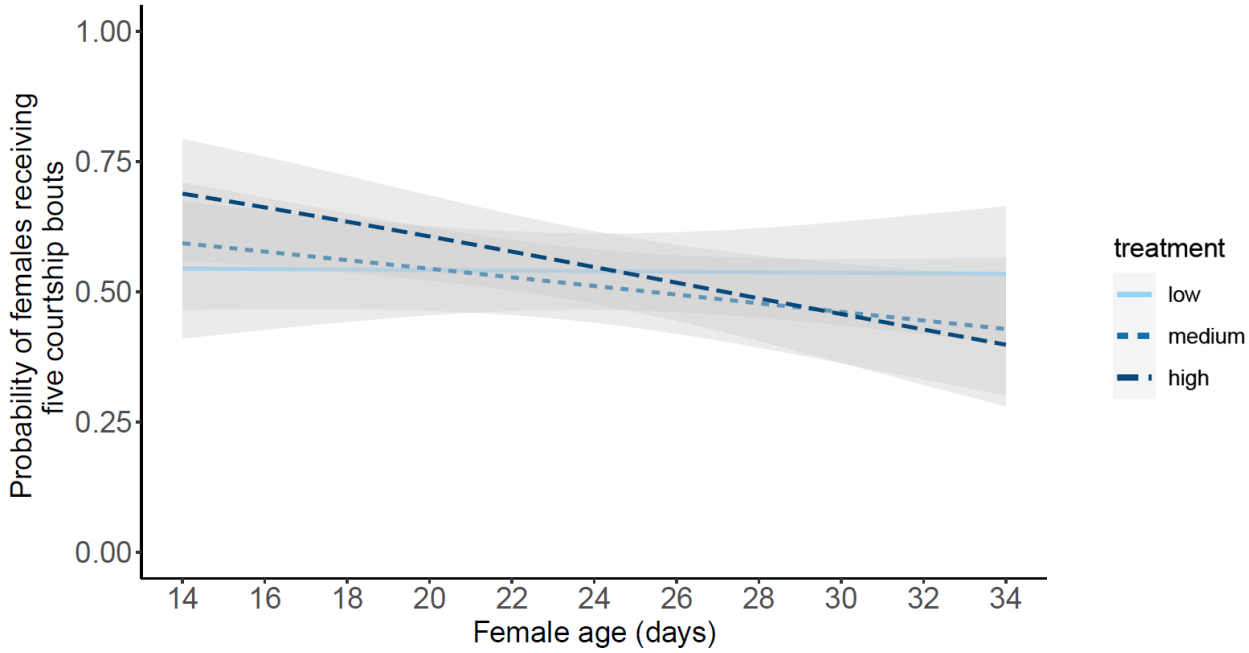

Figure S5.2. Replicate 1 courtship observations. The relationship between female age and attractiveness based on whether females received a minimum of five courtship bouts during their mating or harassment trials. The lines represent predicted probabilities derived from a logistic regression and the grey shaded regions represent 95% confidence intervals. The initial sample sizes for each treatment are N = 18 (low), N = 20 (medium), and N = 20 (high) but decline slightly due to mortality resulting in sample sizes of 17, 16, and 19 for the low, medium, and high treatments, respectively, when females were 34 days old.

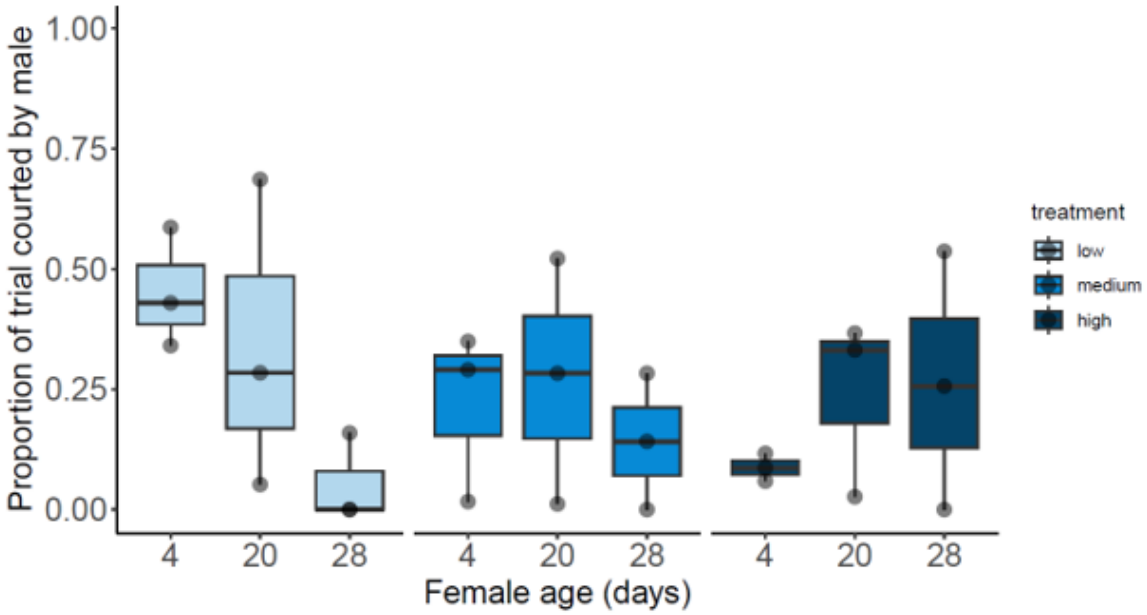

Figure S5.3. Replicate 2 courtship observations. The proportion of total trial duration where females were courted by males. We observed the same three females from each treatment on each of the days we performed courtship trials. Data from females at age 12 were excluded because males in all 9 trials did not court during the 10 min observation trials.

CHAPTER 6 – DISCUSSION

6.1 Overview

In this thesis, I examined the intersection of sexual conflict and social behaviour as well as the fitness consequences of polyandry using two insect species: bed bugs (*Cimex lectularius*) and fruit flies (*Drosophila melanogaster*). I started by testing if female bed bugs exhibited social strategies for mitigating the costs of sexual harassment and costly traumatic insemination. Specifically, I used a social network framework to test if females exhibited decreased sociality compared to males or if were seen aggregating with other females at a higher rate than chance (Chapter 2). Next, based on the rates of insemination I observed in Chapter 2, I explicitly quantified the fitness costs of traumatic insemination (Chapter 3). After showing that observed rates of traumatic insemination are costly to females, I tested the effect of female insemination recency on female avoidance and male rejection behaviours. While recently inseminated females did not avoid males at higher rates, they were more frequently rejected by males. To reconcile the apparent contradiction between the costs of traumatic insemination and no evidence of increased avoidance in recently inseminated females, I tracked the avoidance behaviours of a cohort of female bed bugs as they received six consecutive inseminations. Here, I found that females increase avoidance of males once they have been inseminated three consecutive times and therefore do possess plastic avoidance strategies based on their own sexual history. I then tested how social experience influences male abilities to secure inseminations and females' abilities to mitigate costly insemination (Chapter 4). I found that in bed bugs, social experience does not appear to improve sexual performance in either sex. Finally, using fruit flies, I tested the fitness consequences of polyandry for females, in the absence of excessive sexual harassment, and found that higher mating rates resulted in increased lifetime reproductive success (Chapter 5).

6.2 Female social and behavioural responses to sexual conflict

In the introduction, I broadly defined sexual conflict as instances where the reproductive optima between males and females are not aligned with one another (Parker, 1979). One of the major goals of my dissertation was to examine whether and how sexual conflict can shape the social interactions of animals. I opted to use bed bugs as a model organism for this research, first, because they exhibit social behaviour by forming aggregations (Gries et al., 2015; Pfiester et al., 2009; Reinhardt & Siva-Jothy, 2007), but also because they are one of the most famously cited examples of sexual conflict (Perry & Rowe, 2015; Siva-Jothy, 2006). In my first experiment where I observed groups of individually marked bed bugs in either low or high conflict environments (Chapter 2), I predicted that females might mitigate the costs of harassment from males by either exhibiting a decreased tendency to aggregate with conspecifics or selectivity aggregating with other females. To my surprise, females exhibited higher levels of sociality compared to males and there was also no evidence of aggregations being significantly assorted by sex. This lack of social avoidance at the group level led us to question if natural rates of traumatic insemination are actually costly to females, which I later tested in Chapter 3. But first, I measured the social preferences of females individually and found that females strongly prefer shelters with cues of other females compared to shelters with cues of other males. This discrepancy between females' individual social preferences and the lack of avoidance of males at the group level demonstrates that the sexes can be in conflict over their ideal composition of social groups, thus suggesting sexual conflict over the social environment. These findings also suggest that unlike in other species like cockroaches (*Diploptera punctata*) (Stanley et al., 2018) and Trinidadian guppies (*Poecilia reticulata*) (Darden & Croft, 2008), female bed bugs appear to be incapable of shaping their social environment in a way that reduces their exposure to sexual harassment. Lastly, given that my conclusions were drawn from the combined results of a network-based experiment and individual choice assays, Chapter 2 demonstrate the importance of observing behaviour at both the individual and group level.

While highly insightful, the results from Chapter 2 presented a new paradox; despite the extreme and potentially costly mode of sexual conflict, females experienced traumatic inseminations rather often, even under our semi-naturalistic settings which provided ample evasion and hiding opportunities. Furthermore, previous studies assessing the cost of traumatic insemination for females led to inconsistent results with one experiment showing high costs and another documenting no lifetime fitness costs of traumatic insemination (Morrow & Arnqvist, 2003; Stutt & Siva-Jothy, 2001). Therefore, to continue using bed bugs as a model of extreme sexual conflict, it was crucial that I explicitly quantified the extent to which realistic rates of traumatic insemination reduces female fitness, if at all. Through tracking females that were inseminated at either daily rates or weekly rates, I found that daily rates of traumatic insemination, which reflected the rate of insemination observed in Chapter 2, resulted in a dramatic reduction in females' longevity, egg production and lifetime fitness (Chapter 3). These high costs indicate that male and female optimal rates of traumatic insemination are in fact in conflict with one another and that observed rates of insemination fall closer to the male rather than female optimum.

Because I had previously observed females running away from males in response to mounts, I wanted to explicitly document the behaviour to better understand how females mitigate the costs of excessive mating (Chapter 3). I found that after receiving three consecutive inseminations, females increased the proportion of time they spent running away from males. While female avoidance of males either prior to or during mating has been documented in a handful of other species (Baxter & Dukas, 2017; Crudgington & Siva-Jothy, 2000; Killen et al., 2016), we are, to my knowledge, the first to formally report on this plastic running away response in bed bugs, despite bed bugs being one of the most notable examples of sexual conflict. Moreover, the fact that females spent very little time avoiding males until their fourth daily insemination suggests that up to three inseminations either increases or at least does not substantially decrease female fitness. Thus, while daily insemination should be avoided, mating with up to three males may provide females with key direct or indirect benefits. For example, females may

choose to mate with a small handful of males instead of a single male to guard against cryptic mating failure, which is when copulations do not result in the production of fertile offspring (Greenway et al., 2015; Greenway & Shuker, 2015; Tyler & Tregenza, 2013). Overall, Chapter 3 demonstrates that examining how females respond to males, especially under varying conditions can provide insight into the selective pressures that shape optimal female mating behaviour.

6.3 Social experience and male responses to cues of sperm competition

Social experience is known to greatly alter the behaviour of animals, typically in a way that improves an individual's fitness (Harlow et al., 1965; Hesse & Thünken, 2014; Taborsky et al., 2012). However, I found that social experience did not improve males bed bugs' abilities to secure inseminations or female bed bugs' abilities to avoid inseminations (Chapter 4). Why some species show strong effects of experience while others do not therefore remain unclear and should be examined in further detail (Dukas & Bailey, 2024).

Even though social experience did not improve bed bugs' social competence, my experiments generated several lines of evidence showing that bed bugs are socially perceptive. For example, in Chapter 2, our social preference tests revealed that both male and female bed bugs prefer to occupy shelters with cues of conspecifics over identical shelters with no social cues. Furthermore, male bed bugs appear to be especially sensitive to cues of sperm competition which is likely a direct consequence of the high rates of female multiple mating that we observed. First, in Chapter 2, we found that males can discriminate between cues left by virgin vs. mated females, with a preference for virgin female cues. Next, in Chapter 3, we found that males will outright reject insemination opportunities with recently inseminated females in favour of distantly inseminated females. We also found that males will reduce the amount of ejaculate they invest into females that have been previously inseminated a greater number of times. Lastly, in Chapter 4, we found that males terminated mounts directed at socialized females more frequently than mounts directed at previously isolated females. Here, social vs. isolated

females were matched in their sexual history but socialized females likely had chemical cues indicating prior interactions with rival males. Altogether, these results demonstrate male mate choice in a species that lacks paternal care or elaborate courtship rituals and suggests that the costs of producing ejaculate and mating alone can be nontrivial for males. Across all three of these chapters, males consistently discriminated against recently inseminated females which is a similar pattern that has been reported in the great snipe (*Gallinago media*) (Sæther et al., 2001) and red flour beetles (*Tribolium castaneum*) (Arnaud & Haubruge, 1999). However, some theoretical and empirical studies have shown that males generally benefit from investing more into mated as opposed to virgin females (Parker & Pizzari, 2010; Wedell et al., 2002). Therefore, more experiments are needed to uncover the selective forces that govern sperm allocation and male ejaculate investment strategies across taxa.

6.4 The female fitness consequences of polyandry

Polyandry is common across the animal kingdom which can have important consequences for post-copulatory competition amongst males and the strength of sexual selection on females (Pizzari & Wedell, 2013; Snook, 2014; Taylor et al., 2014). The extent to which polyandry influences sexual selection on females, however, largely depends on the relationship between number of unique mates and fitness for females. In the introduction, I provided an overview of the various costs and benefits that females can accrue from mating and also summarized existing research showing that a single mating typically does not maximize fitness for females (Arnqvist & Nilsson, 2000; Slatyer et al., 2012; South & Lewis, 2011). Importantly though, few studies have assessed higher, more realistic rates of polyandry. In Chapter 3, we saw that daily compared to weekly rates of traumatic insemination drastically reduced female fitness. These findings illustrate that some instances of polyandry may be due to coercion by males and sexual conflict over mating rates. However, in Chapter 5, where I exposed female fruit flies to either low (0.125/day), medium (0.25/day), or high (0.5/day) mating rates, I found that increasing rates of polyandry resulted in increased female fitness. These contradicting findings

indicate that in other species, naturally observed rates of polyandry may be evolutionarily adaptive for females.

6.5 Future Directions

There are several avenues for expanding upon these lines of research which cover a broad range of topics. First, I presented multiple lines of evidence showing that in a highly polyandrous species like the bed bug, the threat of sperm competition can greatly influence male social preferences and sexual behaviour (Chapters 2, 3, and 4). However, asides from one study suggesting weak last male sperm precedence (Stutt & Siva-Jothy, 2001), sperm usage patterns in bed bugs remain poorly understood. It has long been recognized that polyandry has important consequences for sperm competition and sexual selection on males (Kvarnemo & Simmons, 2013; Parker, 1970; Wedell et al., 2002). In fact, female multiple mating can even potentially decouple the relationship between mating success and fitness in males (McDonald & Pizzari, 2016, 2017). However, Greenway et al., (2021) showed that how polyandry influences the direction and strength of sexual selection on males depends largely on a species' sperm use patterns. For these reasons, obtaining more data on sperm usage patterns in bed bugs would be beneficial for furthering our understanding of the traits and behaviours that influence male reproductive success and elucidating how polyandry shapes sexual selection in animals more broadly. During my graduate studies, I attempted to uncover sperm usage patterns in bed bugs for when females are inseminated three consecutive times. Unfortunately, with the sterile insect method that I was using, I was unable to sterilize male bed bugs without also inducing behavioural abnormalities (Yan et al., unpublished data). Therefore, future work should consider the use of molecular tools such as microsatellite markers to uncover patterns of sperm usage and explore other post-copulatory sexual selection mechanisms like sperm competition and cryptic female choice.

In general, combining molecular methods of parentage assignment with a network-based approach where the individuals involved in all mating interactions can be observed under realistic, group conditions would allow researchers to begin unravelling

the complex interplay between precopulatory and postcopulatory sexual selection. With the ability to determine how many offspring each male sires in populations of freely mating individuals, researchers can examine to what extent males experience trade-offs between investing in pre- vs. post-copulatory competition. Such trade-offs have been documented in a handful of other animals like fruit flies and the leaf-footed cactus bug (*Narnia femorata*) (Filice & Dukas, 2019; Joseph et al., 2018). However, in a study on red junglefowl (*Gallus gallus*) that combined molecular parentage data with detailed network-based observations, younger and more aggressive males were found to perform better in both pre- and post-copulatory episodes of sexual selection (McDonald et al., 2017). Therefore, more studies employing this combination of techniques on a variety of species are needed to further disentangle how polyandry shapes the sexual selection on males.

Performing parentage assignment in a species that is easily trackable in the lab like bed bugs can also reveal new insights about how an animal's social network position influences their fitness. Multiple studies have shown that being strongly socially connected is associated with increased longevity and/or reproductive success (Beck et al., 2021; Bzdok & Dunbar, 2020; Formica et al., 2012; Oh & Badyaev, 2010; Turner et al., 2021). However, the extreme sexual conflict seen in bed bugs could potentially de-couple the frequently reported positive association between sociality and fitness seen in most other animals. Therefore, using molecular parentage assignment techniques to measure the direct fitness consequences of increased social connectedness in bed bugs would be fruitful for elucidating how sexual conflict shapes social behaviour and advance our understanding of the evolution of sociality more broadly.

Next, more research is needed to uncover the optimal patterns of mating for females. My results showing that more naturalistic rates of polyandry lead to large fitness reductions in female bed bugs (Chapter 3) but fitness gains in female fruit flies (Chapter 5) demonstrate that we still have a rather narrow understanding of the direct benefits of polyandry. Currently, I am employing a meta-analytical approach to resolve some open questions about polyandry. For example, do females exhibit optimal intermediate mating

rates? Are previous estimates of the cost of mating inflated due to previous insufficient control of sexual harassment from males? And does taxonomic affiliation play a role in whether females benefit from polyandry? While I expect the results of this meta-analysis to be highly informative, my literature searches have revealed that there is still a lack of existing studies assessing the effects of higher, more relevant rates of polyandry which means more experiments across various taxa are needed.

Finally, the extent to which indirect benefits play a role in the evolution and maintenance of polyandry remain unclear. In Slatyer et al. (2012)'s meta-analysis on genetic benefits, polyandry was not shown to enhance any of the offspring traits included in their study (growth rate, survival, adult size). A small handful of experimental studies, however, have shown that polyandry can result in higher quality offspring (Fisher et al., 2006; Ivy & Sakaluk, 2005; Maklakov & Lubin, 2006). Future studies should therefore aim to test whether females gain indirect benefits from mating with multiple males and if so, how these genetic benefits influence female reproductive strategies.

6.6 Conclusion

Taken together, the work presented here on bed bugs and fruit flies provide new insight into the social and sexual lives of animals. In this thesis, I have shown that reproductive conflict between the sexes can shape social dynamics. Additionally, my findings demonstrate that for females, polyandry can have both positive and negative effects on fitness and for males, polyandry can create selective pressures on the ability to detect and plastically respond to cues of sperm competition. I have also introduced methods of employing social network analysis in a way that blends controlled laboratory experiments with ecologically relevant field studies on freely interacting, individually identifiable animals. Altogether, the results from this dissertation contribute to our understanding of sexual conflict, social behaviour, and polyandry and will hopefully pave the way for future research in these areas.

6.7 References

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